OXIDATIVE DAMAGE TO ERYTHROCYTES BY MYELOPEROXIDASE. PROTECTIVE ACTION OF SERUM PROTEINS

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KEY WORDS: oxidation of erythrocytes; myeloperoxidase; anti-oxidative action of serum proteins.

Generation of active forms of oxygen (oxidative burst) by phagocytic cells (neutrophils, macrophages) is one of the mechanisms of protection of the organism against foreign agents. Phagocytes secrete not only oxygen radicals ($O_2^{\cdot}$, H_2O_2, $OH^{\cdot}$), but also hypochlorous acid (HOCl). HOCl is a product of the oxidation reaction of chloride by hydrogen peroxide, catalyzed by myeloperoxidase (MPO) [3]. The importance of MPO for protection of the host organism is great and is due not only to the high concentration (up to 5% of the dry mass of the cell), but also the high nonspecific activation of HOCl in reactions with different biological compounds [2, 13].

In the course of the oxidative burst, reaction intermediates of oxygen not only are secreted into the region of the phagolysosome, but they also enter the immediate microenvironment, where they can induce irreversible damage to tissues, cells, and macromolecules. An additional factor enhancing the negative aspects of phagocytosis, is secretion of large quantities of MPO (more than 20% of its content in the cell) into the extracellular medium [9]. There is much evidence in support of the participation of MPO in damage to various cells (leukocytes, erythrocytes, platelets, fibroblasts, and so on), in the inactivation of of proteins, bacterial toxins, and leukotrienes [8, 12].

Most investigations to demonstrate the toxic effects of MPO have been undertaken under conditions of reaction only between agent and "target". The real situation is more complex, and when injuries arising are evaluated, it is evidently necessary to take into account the presence of various biologically active compounds, capable of influencing the processes taking place.

The writers showed previously that serum proteins can inactivate active forms of oxygen generated by MPO [1]. In the investigation described below the effect of human blood serum proteins on oxidative damage to erythrocytes, taking place as a result of the action of MPO, was studied.

EXPERIMENTAL METHOD

Erythrocytes were sedimented from freshly donated blood by centrifugation at 900g for 10 min at 4°C. The sedimented erythrocytes were washed twice with buffered physiological saline and adjusted to a concentration of $6 \cdot 10^8$ cells/ml. MPO was isolated from hog peripheral blood neutrophils by the method developed by the writers previously [4]. Enzymic activity of MPO, determined relative to oxidation of o-dianisidine, was 55 units/nmole protein.

The MPO and erythrocytes were incubated in 10 mM Na-phosphate buffer, pH 7.4, in the presence of 150 mM NaCl; the concentration of erythrocytes was $6 \cdot 10^7$ cells/ml, and of the enzyme 8 nM; the incubation time was 30 min. H_2O_2 was generated in the medium in a glucose oxidase-glucose system. The reaction was triggered by the addition of glucose oxidase to a final concentration of $17 \cdot 10^{-3}$ units/ml. After the end of incubation the erythrocytes were sedimented at 900g for 10 min at 4°C. Concentrations of oxy-, deoxy-, and methemoglobin were determined in the supernatant and the lysed residue by the method in [5]. The degree

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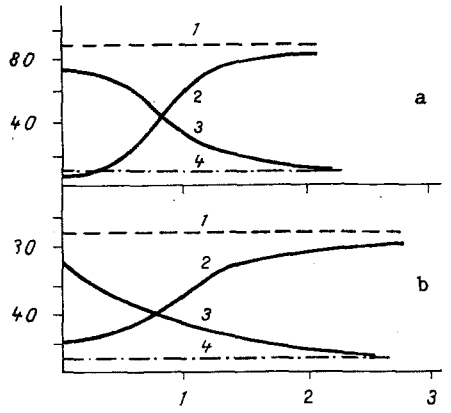

Fig. 1

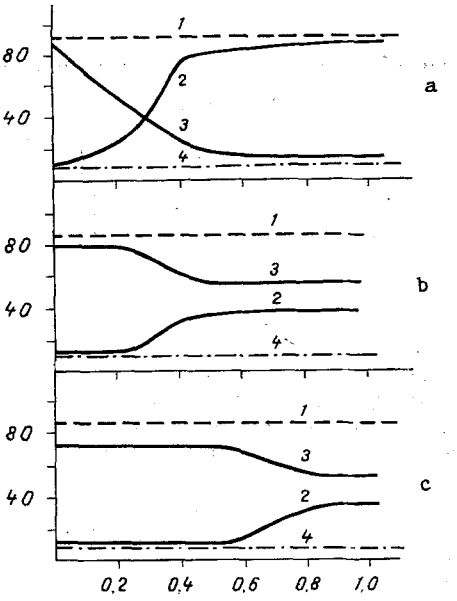

Fig. 2

Fig. 1. Concentrations of met- (2) and oxyhemoglobin (3) in erythrocytes treated with MPO, on the addition of various concenrations of serum proteins. a) Albumin, b) immunoglobulin; 1 and 4 - levels of oxy- and methemoglobin in untreated erythrocytes respectively. Here and in Fig. 2: abscissa, protein concentration (mg/ml); ordinate, concentration of hemoglobins (%).

Fig. 2. Concentration of oxy- (2) and methemoglobin (3) in erythrocytes exposed to the action of MPO, after addition of different concentrations of antioxidant proteins. a) CP; b) TF; c) SOD. 1 and 4) Levels of oxy- and methemoglobin respectively in untreated erythrocytes.

of lysis of the erythrocytes (K) was determined by measuring the change in scatter of light at 740 nm by the equation:

$$K = 1 - \frac{A_{740} - B_{740}}{C_{740} - B_{740}},$$

where A_{740} is the optical density of the suspension of oxidized erythrocytes in phosphate buffer, B_{740} the optical density of a suspension of erythrocytes lysed in water, and C_{740} the optical density of a suspension of untreated erythrocytes in phosphate buffer.

The spectral characteristics of the supernatant and of the cell lysates were recorded on a Hitachi-330 dual-beam spectrophotometer (Japan).

EXPERIMENTAL RESULTS

Incubation of erythrocytes in medium containing an MPO-H_2O_2-Cl^- system led to cell damage. Partial lysis of erythrocytes took place with oxidation of hemoglobin, leading to the appearance of the oxidized form (methemoglobin) and an insoluble intracellular product.

Addition of the basic serum proteins (albumin and immunoglobulin G) to the incubation medium inhibited oxidation of hemoglobin. Dependence of the degree of inhibition of oxidation on protein concentration is complex in character (Fig. 1). A region of concentrations can be distinguished in which proteins do not inhibit oxidation and do not play the role of traps of products of myeloperoxidase catalysis. For albumin and immunoglobulin G these concentrations are under 1 mg/ml. There exists a narrow range of protein concentrations in which a sharp increase is observed in the ability of proteins to inhibit intracellular oxidation of hemoglobin, induced by MPO. With a further increase in the protein concentration,

TABLE 1. Effect of Hypochlorite and MPO on Lysis of Erythrocytes (M ± m)

Oxidative system	Degree of lysis, %
MPO-H_2O_2-Cl^-	5±1
HOCl 10 μm	0
50 μm	0
100 μm	72±5
200 μm	75±6
400 μm	100±7
800 μm	100±5

virtually complete protection of the erythrocytes against oxidation is achieved; these concentrations, moreover, are below the protein concentrations in the serum. It will be noted that this "sigmoid" relationship points to the cooperative character of the processes taking place in the system studied under the particular conditions which exist there. Concentration dependences, similar in character, were observed also for antioxidant proteins: ceruloplasmin (CP), transferrin (TF), and superoxide dismutase (SOD) (Fig. 2). CP was the most active in preventing oxidation of hemoglobin. Low activity, even compared with albumin and IgG, was exhibited by TF and SOD, although traditionally they are regarded as elements of the host's antioxidative system.

Serum proteins, actively influencing oxidation of the intracellular contents of the erythrocytes, do not affect lysis of erythrocytes, which in the presence of MPO, takes place only to a very insignificant degree (Table 1). Even in conditions when hemoglobin is converted almost completely into methemoglobin, no increase was observed in the degree of lysis compared with the control. Weak lytic activity characteristic of MPO also was observed in the case of contact of erythrocytes directly with stimulated neutrophils [5]. On the other hand, incubation of erythrocytes with HOCl led to considerable lysis, the degree of lysis being a stepwise function of the hypochlorite concentration, evidence of the cooperative nature of the process (Table 1). Under the influence of HOCl as an oxidizing agent, intensive oxidation of hemoglobin was observed only in above-lytic concentrations (over 50 μM). The reasons for the differences in the mechanism of action of exogenous hypochlorite and hypochlorite produced during myeloperoxidase catalysis are not clear.

Despite the fact that the level of antioxidative enzymes (glutathione peroxidase, SOD, catalase) in the extracellular medium is low [6], serum possesses considerable antioxidative activity is exhibited against peroxide radicals [11] and in reactions of inhibition of $O_2^{\cdot}$ and H_2O_2 dependent formation of $OH^{\cdot}$ radicals [7]. It has been suggested that the serum proteins make an important contribution to these diverse antioxidative functions [7, 11]. The ability of serum proteins to scavenge active forms of oxygen produced in the course of myeloperoxidase catalysis was reported by the writers previously [1]. It was suggested that the principal serum proteins (albumin, IgG) may be physiological inhibitors of the myeloperoxidase reaction. It was later shown [10] that albumin, CP, and TF protect α-1-antiprotease against oxidative inactivation by hypochlorite, confirming our own conclusions.

Thus the previous suggestion that serum proteins (albumin, IgG, CP and, evidently, others also) can play the role of elements of the "antioxidative buffer" system of the host organism is thus confirmed not only at the molecular level [1, 7, 10, 11], but also at the level of the whole cell (erythrocyte).

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EXTERNAL OXIDATION PATHWAY IN NERVE TISSUE

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Besides the main respiratory chain, located in the inner membrane, intact mitochondria of the liver and heart are also known to have an additional electron-transport system for oxidation of extramitochondrial NADH, including flavine-containing NADH-cytochrome c-reductase of the outer membrane, and cytochrome b_5 and the labile fraction of cytochrome c, located in the inter membranous space. This redox chain shunts the flow electrons to the cytochrome oxidase of the inner mitochondrial membrane. The external oxidation pathway of NADH is resistant to amobarbital and antimycin A but sensitive to KCN and to low concentrations of mersalyl; it is activated under conditions leading to swelling and approximation of the membranes, and has been identified not only in the liver and heart, but also in mitochondria of skeletal muscles and in microorganisms, yeasts, and molds [3, 5-7, 9-14]. However, it is not yet clear whether this pathway functions in the intact mammalian cell.

The aim of this investigation was to study the presence and role of the external oxidation pathway in brain tissue under different conditions of oxygenation.

EXPERIMENTAL METHOD

Experiments were carried out on brain sections from noninbred male albino rats weighing 160-200 g, divided beforehand into those with high (HR) and low (LR) resistance to hypoxia. Sections were cut by the standard method on a microtome. Their respiration rate was determined polarographically [2]. To assess the relative contribution of the external oxidation pathway (EOP) to total respiration, mersalyl (10^{-5} M), a specific inhibitor of this pathway, was used. The concentration of the inhibitor was chosen on the basis of data in the literature [6, 13]. Our results obtained on isolated brain mitochondria are evidence that this concentration of mersalyl has no significant effect on the basic respiratory chain. Higher concentrations of mersalyl (10^{-4} M) inhibited mitochondrial respiration. The effect of inhibitors of various regions of the basic respiratory chain (amobarbital, malonate, antimycin A, KCN) on the mersalyl-sensitive component of respiration (MSR) of the brain preparations was estimated on the basis of its change under the influence of these inhibitors.

EXPERIMENTAL RESULTS

MSR in brain slices oxidizing glucose in carbogen-containing medium ("normoxic" conditions) accounted for 20-25% of the total tissue O_2 consumption (Table 1). The MSR of the brain was resistant to amytal and antimycin A and virtually completely inhibited in the presence of KCN (Table 1). This is evidence that the brain contains and EOP characterized by

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