

Original Contribution

Hemoglobin autoxidation and regulation of endogenous H_2O_2 levels in erythrocytes[☆]

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Abstract

Red cells from mice deficient in glutathione peroxidase-1 were used to estimate the hemoglobin autoxidation rate and the endogenous level of H_2O_2 and superoxide. Methemoglobin and the rate of catalase inactivation by 3-amino-2,4,5-triazole (3-AT) were determined. In contrast with iodoacetamide-treated red cells, catalase was not inactivated by 3-AT in glutathione peroxidase-deficient erythrocytes. Kinetic models incorporating reactions known to involve H_2O_2 and superoxide in the erythrocyte were used to estimate H_2O_2 , superoxide, and methemoglobin levels. The experimental data could not be modeled unless the intraerythrocytic concentration of Compound I is very low. Two additional models were tested. In one, it was assumed that a rearranged Compound I, termed Compound II*, does not react with 3-AT. However, experiments with an NADPH-generating system provided evidence that this mechanism does not occur. A second model that explicitly includes peroxiredoxin II can fit the experimental findings. Insertion of the data into the model predicted a hemoglobin autoxidation rate constant of $4.5 \times 10^{-7} \text{ s}^{-1}$ and an endogenous H_2O_2 and superoxide concentrations of 5×10^{-11} and $5 \times 10^{-13} \text{ M}$, respectively, lower than previous estimates.

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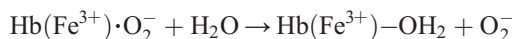
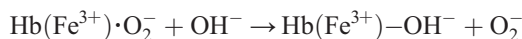
Keywords: Erythrocyte; Oxidative defense; Hemoglobin; Gene deletion; Glutathione; Glutathione peroxidase; Catalase; ROS; Free radicals

Introduction

Reactive oxygen species (ROS) are of increasing interest as agents of pathological changes in aging and a variety of disease states, including atherosclerosis [1], Parkinson's disease [2], and diabetes [3], among others. In most cells, the major source of ROS is the mitochondrion [4]. The red cell is different in this regard. Lacking mitochondria, its major source of ROS is the oxygen carrier protein hemoglobin (Hb), which undergoes autoxidation to produce

superoxide [5,6]. Since the intraerythrocytic concentration of oxygenated Hb is 5 mM, even a small rate of autoxidation can produce substantial levels of ROS. It is of interest to determine the extent of this oxidant challenge and the resulting levels of ROS in the erythrocyte. The oxidative stress may be adequate to damage the red cell itself, and, because red cells make up 40% of the blood volume, ROS escaping from the red cell have the potential to damage other components of the circulation.

The current picture [6–9] of oxidant reduction and elimination in the red cell involves Hb, glutathione peroxidase (GSHPx), and superoxide dismutase (SOD). Hb autoxidation follows the polarization of the Fe–oxygen bond. The overall reaction is a bimolecular substitution reaction in which water or hydroxide replaces the oxygen [10]:



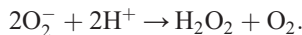
Abbreviations: ROS, reactive oxygen species; Hb, hemoglobin; GSHPx, glutathione peroxidase; SOD, superoxide dismutase; 3-AT, 3-amino-2,4,5-triazole; IAA, iodoacetamide; metHb, methemoglobin; Prx, peroxiredoxin.

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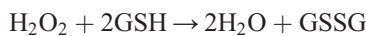
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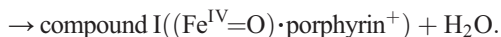
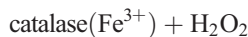
Since the red cell has high levels of superoxide dismutase, the O_2^- produced will be converted to H_2O_2 :



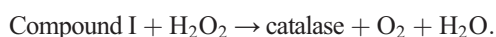
H_2O_2 is eliminated by glutathione peroxidase



and catalase. The reaction mechanism of catalase has two steps [11]. In the first, catalase transfers two electrons to H_2O_2 , forming Compound I, in which the active site heme iron is oxidized to ferryl Fe, and the porphyrin moiety has lost an electron:



Compound I is then reduced by a second H_2O_2 , restoring the ground state:



Giulivi et al. [12] proposed a method to determine the intracellular rate of Hb autoxidation and the concentrations of H_2O_2 and $O_2^{\bullet-}$ in the red cell. Catalase Compound I is specifically and irreversibly inhibited by covalent reaction with 3-amino-2,4,5-triazole (3-AT) [13,14]. Thus, the rate of catalase inactivation by 3-AT should be related to H_2O_2 levels. It was found, however, that 3-AT did not inhibit catalase in unperturbed erythrocytes [12,15], implying that no H_2O_2 was produced. To explain this surprising result, Giulivi and co-workers noted an early proposal by Hochstein and co-workers that intraerythrocytic GSHPx is the major H_2O_2 -catabolizing enzyme in the red cell [16]. Thus, they proposed that little Compound I will form because GSHPx maintains a low erythrocytic H_2O_2 level. To test this hypothesis, cells were pretreated with iodoacetamide (IAA) to eliminate GSH and prevent GSHPx reduction of H_2O_2 . They found that in these IAA-treated cells, catalase was inhibited by 3-AT, supporting their model.

We have repeated these measurements, taking advantage of the availability of red cells from mice with a disrupted GSHPx-1 gene [17,18]. These red cells are devoid of GSHPx activity [18], and can be used to reexamine the question of erythrocyte autoxidation and H_2O_2 , without the necessity for IAA treatment. We find that catalase is inhibited very slowly by 3-AT in GSHPx-deficient mouse red cells. This observation, and additional data obtained with (Cu,Zn)-superoxide dismutase-deficient erythrocytes, suggests that the intraerythrocytic levels of ROS are lower than previously thought. A kinetic model of H_2O_2 and $O_2^{\bullet-}$ metabolism in the red cell which explicitly includes peroxiredoxin activity can fit the experimental data.

Materials and methods

Knockout mice

The generation of mice deficient in each of the following enzymes has been described: GSHPx-1 [17], (Cu,Zn)-

superoxide dismutase [19], and catalase [20]. As controls, matched wild-type mice on a 129SV/B6 genetic background were used in this study.

Erythrocytes

For these experiments, blood was obtained from the hearts of enzyme-deficient mice and matched controls after Nembutal anesthesia. Human red cells were obtained from normal volunteers. The erythrocytes were washed twice in PBS (145 mM NaCl, 5 mM NaPi, 1 mM EDTA, pH 7.4) and white cells were removed by filtration through cellulose [21].

Enzyme assays

The absence of enzymatic activity was verified in the enzyme-deficient cells. For glutathione peroxidase activity, cellulose filtered cells were assayed as described by Beutler [22], with one modification. To eliminate interference from methemoglobin [23], 10 mg NaCN and 30 mg $K_3Fe(CN)_6$ were added to 10 ml of the 1 M Tris, 5 mM EDTA, pH 8.0, buffer used in the assay. (Cu,Zn)-SOD was assayed as described by Beutler [22].

Catalase was assayed by the method of Chance [24], as modified by Aebi [25]. A spectrophotometer (Pharmacia Ultrospec III) was zeroed at 240 nm with a blank composed of 100 μ l hemolysate with 900 μ l of Pi buffer (50 mM sodium phosphate, pH 7.0). For the assay, 100 μ l hemolysate was mixed with 567 μ l of Pi buffer, and the reaction was started by the addition of 333 μ l of 30 mM H_2O_2 in buffer. Readings were obtained at 15 and 30 s, and the first-order rate constant was calculated. To estimate the catalase concentration, it was assumed that the specific activity of mouse catalase is similar to that of human, and a catalytic constant of $3.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ was used. The volume of red cells in the assay was calculated by measuring hemolysate hemoglobin, and using a value of 336 g/L for the hemoglobin concentration in the erythrocyte of the C57BL mouse [26].

Other assays

H_2O_2 was assayed by the method of Green and Hill [27]. The concentration of H_2O_2 in the stock solution was quantitated [28] using an A_{240} value of $43.6 \text{ M}^{-1} \text{ cm}^{-1}$. 3-AT was assayed by the method of Agrawal and Margoliash [29]. GSH was determined as described earlier [30]. Methemoglobin (metHb) was determined by a standard method [31] or spectroscopically [5]. Unless otherwise noted, reagents were obtained from Sigma.

Calculations

The simultaneous differential equations for the models were solved using the Chemical Reactions module of Berkeley Madonna [32].

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