Enhancement of Erythrocyte Superoxide Dismutase Activity: Effects on Cellular Oxidant Defense

By Mark D. Scott, John W. Eaton, Frans A. Kuypers, Daniel T.-Y. Chiu, and Bertram H. Lubin

To delineate further the role of superoxide dismutase (SOD) in red blood cell (RBC) oxidant defense, normal human erythrocytes were osmotically lysed and resealed in the presence of varying concentrations of exogenous SOD. This resulted in a dose-dependent increase in SOD activity in the resealed erythrocytes while maintaining nearly normal RBC hemoglobin concentration (~10% decrease from the control value), cell volume, and cellular deformability. Surprisingly, a five- or ninefold increase in SOD activity yielded no additional protection against superoxide-generating drugs (phenazine methosulfate or menadione sodium bisulfite). No significant differences were observed between the control and SOD-loaded RBCs in O₂⁻-driven methemoglobin formation or generation of thiobarbituric acid-reactive substances. In contrast, RBCs with elevated SOD activity pretreated with sodium azide (to block catalase activity) or 1-chloro-2,4-dinitrobenzene (to deplete reduced glutathione, GSH) showed significantly enhanced methemoglobin generation in response to superoxide-generating drugs. No differential response was noted between the control, control-ressealed, and SOD-loaded RBCs to oxidants other than superoxide. Based on our results and other data, we conclude that elevated SOD activity may imbalance cellular oxidant defense, resulting in enhanced oxidation due to the accelerated generation of H₂O₂, the product of O₂⁻ dismutation. This effect is significantly exacerbated under conditions in which H₂O₂ catabolism is altered.

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METHODS

After informed consent was obtained, normal RBCs were collected in heparinized tubes from healthy laboratory volunteers and used within 24 hours of collection. SOD, phenazine methosulfate (PMS), menadione sodium bisulfite, H₂O₂, 1-chloro-2,4-dinitrobenzene, t-butyl hydroperoxide, and fluorescein isothiocyanate (FITC) labeled dextran [FITC-Dextran, average molecular weight (mol wt) 70 kDa] were obtained from Sigma Chemical Co (St Louis, MO). Cumene hydroperoxide was purchased from Aldrich Chemical Co (Milwaukee, WI). Using a modification of previously described dialysis methods,⁴⁶ RBCs were loaded by combining 2 to 5 mL washed, packed RBCs (pRBCs; 80% to 85% hematocrit) and the constituents to be loaded (SOD or GSH) in a test tube with subsequent mixing. The proteins were suspended in 200 to 400 μL isotonic saline before addition to the pRBCs. The test tube contents were sealed in 11.5-mm diameter dialysis tubing (mol wt cutoff of 3,500 daltons) while a high surface/volume ratio was maintained, thereby insuring complete lysis and rescaling. The samples were dialyzed against 1 L lysis buffer containing 5 mmol/L potassium phosphate (pH 7.4) and 2

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mmol/L EDTA at 4°C for 60 minutes with constant mixing of the lysis buffer. The dialysis tubing was transferred to 1 L resalting buffer containing 160 mmol/L NaCl, 5 mmol/L glucose, and 5 mmol/L potassium phosphate (pH 7.4) and mixed for 30 minutes at room temperature. After resalting, cells were washed with saline until the supernatant was clear (five to seven times). Unless otherwise noted, the RBCs were resuspended to a 30% hematocrit in Ca2+- and Mg2+-free Hanks' balanced salt solution (HBSS, GIBCO Laboratories, Grand Island, NY) supplemented with 5 mmol/L glucose. The completeness of RBC osmotic lysis and subsequent incorporation of exogenous compounds was examined by the entrapment of FITC-labeled dextran (70 kd). RBC fluorescence was determined by microscopic analysis and by a fluorescence activated cell sorter (FACS; Becton Dickinson, Mountain View, CA).

To determine whether depletion of small metabolic cofactors (e.g., GSH, NADH, NADPH, and ATP) might occur during the loading procedure, in some experiments 2 or 4 mmol/L GSH (final concentration) was added to the pRBCs before lysis. The intracellular GSH concentration of RBCs ressealed with and without added GSH was then determined by the method of Beutler and expressed as micromoles per gram of hemoglobin.

Mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), and RBC distribution width (RDW) of control and ressealed RBCs were analyzed with an automated Coulter Counter, and cellular morphology was examined by light microscopy. Hemolysates were assayed for SOD activity by the cytochrome c reduction method, and catalase activity was determined by the spectrophotometric disappearance of H2O2. SOD activity is expressed as units of SOD per gram of hemoglobin and is assay system dependent. Catalase activity is expressed as international units per gram of hemoglobin.

The effect of lysis/resalting on RBC deformability was examined with a Technicon Ektacytometer (Technicon Instruments, Tarrytown, NY). Ektacytometry is a sensitive method for detecting changes in osmotic fragility, cell hydration, and cellular deformability. Recently, we modified this technique to allow for continuous monitoring of oxidant-mediated changes in cellular deformability (manuscript submitted). This method allows detection of oxidant-mediated damage before substantial thiobarbituric acid-detectable lipid peroxidation or RBC lysis occurs. RBCs are diluted to a 1% hematocrit in 290 mOsm polyvinylpyrrolidone (Sigma, average mol wt 360,000) and then challenged within the ektacytometer with 1 mmol/L (final concentration) H2O2, cumene- or t-butyl-hydroperoxide. The resultant loss of cellular deformability was continuously monitored and expressed as percentage of initial cellular deformability as a function of time.

Intracellular generation of O2·− was produced by incubating the RBC suspensions with PMS or menadione sodium bisulfite. PMS generates O2·− through an intracellular reaction NADH, whereas menadione sodium bisulfite reacts directly with oxyhemoglobin to produce methemoglobin and O2·−.

Nitroblue tetrazolium (NBT) was used as a qualitative indicator of O2·− concentration. Four O2 are required to reduce NBT to the compound measured by this method. Washed RBCs (control, control-ressealed, and SOD-loaded) were resuspended to a 4% hematocrit in HBSS containing 50 μmol/L NBT. Samples were incubated at 37°C, and 1-mL aliquots were removed at the indicated time points. RBCs were sedimented by centrifugation (1,000 g), and the absorbance of reduced NBT in the supernatant was measured at 560 nm.

The response of control, control-ressealed, and SOD-loaded RBCs to O2·− was assessed by methemoglobin formation and generation of thiobarbituric acid-reactive substances (TBARS) in response to 50 μmol/L PMS and 0.5 mmol/L menadione sodium bisulfite. In some experiments, the control, control-ressealed, and SOD-loaded (4 mg/L pRBCs) RBCs were treated with 5 mmol/L sodium azide to inhibit catalase activity. Although azide inhibits Cu/ZnSOD, the concentration used causes less than 5% inhibition. In additional experiments, a 10% hematocrit of control, control-ressealed, and SOD-loaded RBCs were incubated at 37°C for 1 hour with 2 mmol/L 3-chloro-2,4-dinitrobenzene (CDNB) added to deplete RBC GSH. After CDNB treatment, the cells were washed three times in saline and reduced GSH levels in RBCs were assayed as described to insure GSH depletion. RBC lysis was determined by quantification of released hemoglobin in the supernatant by ferricyanide-cyande (Drabkin's) reagent. Hemoglobin oxidation was determined by spectrophotometric analysis (500 to 700 nm); the concentration of oxy-, met-, and hemichrome hemoglobin was calculated according to the method of Winterbourn. Hemoglobin degradation was determined by following the loss of heme absorbance. The cellular response to O2·− independent oxidants was assessed via sensitivity to H2O2, cumene- and t-butyl-hydroperoxide.

RESULTS

In contrast to other methods for RBC loading, MCV and MCHC (Table 1) are not appreciably altered as a consequence of the dialysis lysis/resalting procedure outlined in this study. The MCH, while significantly altered (P < .05), was still within 10% of control values. The RDW of the ressealed RBCs was also increased (P < .05), indicating a more heterogeneous population. The altered RDW appears to result from formation of small membrane fragments. The observed increase in RDW could be reduced by aspirating the top layer of RBCs during the initial washing after resalting. Examination of RBC morphology demonstrated that these cells exhibit near normal morphology (not shown). Endogenous SOD and catalase activities in the control-ressealed RBCs were 95% and 107%, respectively, that of the control RBCs (Table 2).

This method was shown to be an efficacious means of supplementing RBC antioxidant enzymes in a dose-dependent manner (Table 2). Addition of 4 or 8 mg SOD (3,500 U SOD/mg protein) per milliliter/pRBCs resulted in a 5.1- and ninefold increase, respectively, in activity over control and control-ressealed RBCs. Previously reported encapsulation percentages for human erythrocytes have usually ranged from 30% to 40%,5, with a maximum theoretical entrapment of 60%, of the exogenous compound. Using the method described in this study, approximately 30% of the exogenous SOD was entrapped within the ressealed RBCs. In addition, more than 96% of the ressealed cells incorporated exogenous compounds, as shown by FACS analysis of FITC-dextran entrapment (data not shown). Importantly, addition of SOD or FITC-dextran to intact RBCs, followed by washing in HBSS, did not increase SOD activity (Table 2) or RBC fluorescence, indicating that simple extracellular adherence of the added compounds did not account for the increased enzymatic activity or cellular fluorescence.

The lysis/resalting procedure was also found to have little effect on the concentration of small intracellular molecules such as GSH. After the resalting procedure, GSH concentrations in the control-ressealed and SOD-loaded RBCs were approximately 86% that of control cells (Table 2). Inclusion of 2, 4, or 8 mmol/L GSH in the dialysis tubing...
during lysis and resealing resulted in substantial increases (approximately 130%, 211%, and 345%, respectively) in GSH concentration over control RBCs (Table 2).

Ektacytometric analysis of cellular deformability (DI) showed that the control-resealed and SOD-loaded RBCs maintained near-normal cellular deformability and osmotic fragility in response to an osmotic gradient (Fig 1). The DI<sub>max</sub> (the maximum deformability at 290 mOsm/kg) of the control-resealed and SOD-loaded RBCs were 87.6% ± 8.1% (n = 6) and 90.4% ± 4.7% (n = 6), respectively, that of normal RBCs. Similarly, the control, control-resealed, and SOD-loaded RBCs showed a virtually identical rate in loss of cellular deformability in response to oxidant challenge with H<sub>2</sub>O<sub>2</sub> (Fig 2). The half-maximal deformability (DI<sub>50</sub>) time required for a 50% decrease in the maximum DI) of the control-resealed and SOD-loaded RBCs exposed to H<sub>2</sub>O<sub>2</sub> was 108.3% ± 7.4% (n = 3) and 109.8% ± 13.0% (n = 3), respectively, of the control DI<sub>50</sub>. Similar responses in the DI<sub>50</sub> of the control, control-resealed, and SOD-loaded RBCs to cumene- and t-butyl-hydroperoxide were noted (data not shown). As shown in Fig 2, the slight decrease in GSH content noted in RBCs resealed in the absence of exogenous GSH had no significant effect on RBC oxidant sensitivity to H<sub>2</sub>O<sub>2</sub>

SOD-loading of RBCs resulted in an enhanced O<sub>2</sub>·− scavenging capacity. As shown in Fig 3, the high SOD RBCs showed a significant (~30%; P < .01) decrease in O<sub>2</sub>·−-mediated NBT reduction. This indicates that SOD was efficient in reducing intracellular O<sub>2</sub>·− concentrations. However, challenge with 50 μmol/L PMS resulted in no differential methemoglobin formation (Fig 4) or TBARS generation (Fig 5) in the control, control-resealed, or SOD-loaded RBCs. Hence, even a ninefold increase in SOD activity gave no protection against the toxicity of PMS, a potent O<sub>2</sub>·− generator. Similar results were found in response to menadione sodium bisulfite (data not shown). Both methemoglobin generation and TBARS values were consistently, although not significantly, higher in the SOD-loaded RBCs in response to O<sub>2</sub>·− challenge. Hemoglobin absorption spectra showed that no substantial hemoglobin degradation (as indicated by loss of heme absorbance) occurred as a result of O<sub>2</sub>·− challenge.

However, inhibition of catalase activity by azide (5 mmol/L) or the depletion of reduced GSH by CDNB (2 mmol/L), thereby increasing intracellular H<sub>2</sub>O<sub>2</sub> concentration, resulted in significant increases in methemoglobin generation in the SOD-loaded RBCs as compared with the control cells (Fig 6). This effect is probably a result of the enhanced generation of H<sub>2</sub>O<sub>2</sub> in the high SOD RBCs. Azide treatment in the absence of oxidant stress resulted in no substantial or differential formation of methemoglobin over the time-course of the experiment. CDNB treatment, although generating substantial methemoglobin, resulted in no differential rate of methemoglobin generation in the absence of oxidant stress. Methemoglobin concentrations were significantly (P < .05) higher in the CDNB-treated cells that had undergone osmotic lysis and resealing. Without azide or CDNB treatment, the bolus addition of H<sub>2</sub>O<sub>2</sub> had no differential effect on methemoglobin or TBARS generation in control, control-resealed, and SOD-loaded RBCs (data not shown).

### DISCUSSION

Complex aerobic organisms have assured an adequate and continuous flow of oxygen to their tissues, while simultaneously protecting themselves from the inherent toxicity of oxygen, by two mechanisms: (a) development of specialized oxygen-carrying proteins such as hemoglobin, and (b) concurrent evolution of efficient oxidant defense systems. Previous attempts to understand the role of intracellular antioxidants in preventing oxidant-mediated damage in RBCs have relied on such methods as selective inactivation of antioxid-

### Table 1. Effect of Resealing on Red Cell Parameters

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>MCV</th>
<th>MCH</th>
<th>MCHC</th>
<th>RDW</th>
</tr>
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<tr>
<td>Control</td>
<td>7</td>
<td>88.9 ± 1.5</td>
<td>30.5 ± 0.8</td>
<td>34.2 ± 0.7</td>
<td>12.9 ± 0.2</td>
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<tr>
<td>Control-resealed</td>
<td>7</td>
<td>85.8 ± 4.5</td>
<td>28.5 ± 2.7*</td>
<td>33.2 ± 1.7</td>
<td>16.0 ± 4.5</td>
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<tr>
<td>SOD-loaded</td>
<td>5</td>
<td>86.1 ± 5.4</td>
<td>27.9 ± 3.2*</td>
<td>32.3 ± 2.0*</td>
<td>17.3 ± 5.3*</td>
</tr>
</tbody>
</table>

*Significantly different from control value, P < .05.

### Table 2. Loading of Exogenous Antioxidants into Normal Human Erythrocytes

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control + SOD*†</th>
<th>Resealed</th>
<th>GSH 2 mmol/L</th>
<th>GSH 4 mmol/L</th>
<th>GSH 8 mmol/L</th>
<th>SOD 4 mg*</th>
<th>SOD 8 mg*</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Cat. (IU/g Hb)</td>
<td>154,100 ± 21,900</td>
<td>147,600</td>
<td>164,800 ± 50,360</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>126,500 ± 15,800</td>
<td>128,770 ± 24,440</td>
</tr>
<tr>
<td>SOD (IU/g Hb)</td>
<td>3,194 ± 171</td>
<td>3,088 ± 53</td>
<td>3,030 ± 86</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>16,300 ± 2,280</td>
<td>28,770 ± 2,440</td>
</tr>
<tr>
<td>GSH (μmol/g Hb)</td>
<td>6.4 ± 0.5</td>
<td>6.1 ± 0.2</td>
<td>5.5 ± 0.3</td>
<td>8.5 ± 0.9</td>
<td>13.5 ± 0.8</td>
<td>22.1 ± 0.2</td>
<td>5.4 ± 0.7</td>
<td>5.3 ± 0.4</td>
</tr>
</tbody>
</table>

Abbreviations: Hb, hemoglobin; ND, not determined.

*Per milliliter RBCs.
†Four milligrams SOD per milliliter RBCs.
‡Significantly different from control RBCs, P < .001.
Fig 1. Osmotic lysis-resealing of RBCs has only a minor effect on cellular deformability. Shown is the ektacytometric response of control (curve 1), control-resealed (curve 2), and SOD-loaded (curve 3) RBCs to an osmotic gradient.

Fig 2. Reversible lysis of RBCs does not affect the H$_2$O$_2$-mediated loss of cellular deformability in control and resealed erythrocytes. Control and resealed RBCs were challenged with 1 mmol/L H$_2$O$_2$ with and without sodium azide (1 mmol/L). Azide was used to inactivate catalase irreversibly. H$_2$O$_2$ had little effect on RBCs without azide, but resulted in rapid loss of cellular deformability in azide-treated RBCs.

Fig 3. PMS-mediated generation of superoxide in control and resealed RBCs. The superoxide-dependent reduction of NBT was followed at 560 nm. In the absence of PMS (●), all three samples showed equivalent rates of NBT reduction; the values shown are the mean absorbance ± SD of the control, control-resealed, and SOD-loaded RBCs. Addition of 50 µmol/L PMS resulted in substantial increases in NBT reduction in all three samples. However, the SOD-loaded (▲; 4 mg/mL pRBCs) erythrocytes showed a significantly lower rate of NBT reduction as compared with either the control (○) or control-resealed (●) RBCs. Results are the mean ± SD of four samples from two independent experiments.
dant enzymes, enzyme loading of membrane ghosts, and enzyme augmentation of normal RBCs through reversible lysis.

Several RBC antioxidants can be readily inactivated by chemical means. Catalase is known to be inactivated by cyanide, azide, and 1,2,4-aminotriazole. Attempts to inactivate SOD selectively have involved use of diethyldithiocarbamate. However, all these agents are known to interact with other biologic compounds. Cyanide and azide react with innumerable RBC components, whereas aminotriazole reacts with other heme-containing compounds and requires the presence of H₂O₂. Diethyldithiocarbamate virtually depletes the cell of GSH at the levels necessary to inhibit RBC SOD. Consequently, it is difficult to distinguish the specific roles of the various antioxidants in protecting the RBC from oxidant stress.

Other studies have used RBC ghosts, or artificial liposomes, to study the effects of oxidants and antioxidants on membrane integrity and composition. In general, these articles have reported modest protective effects by low levels of SOD, catalase, or both. Putative hydroxyl radical scavengers such as mannitol have been effective only at relatively high concentrations. However, hydroxyl radicals generated at the membrane surface or within the membrane would not be efficiently scavenged by these hydrophilic scavengers. Furthermore, membrane ghosts and liposomes are not representative of the intact RBC because hemoglobin, a potential "biological Fenton reagent," is absent.

Attempts to augment the antioxidant status of RBCs have been equally unsuccessful. Simple addition of antioxidants to the extracellular space will not adequately protect against intracellular events. Efforts to enhance intracellular enzymes or substrates have generally relied on loading macromolecules into RBCs by hypotonic shock, which opens membrane pores through which molecules can enter. This is followed by subsequent resealing in isotonic buffers, resulting in intracellular entrapment of the enzyme(s). However, early studies resulted in RBCs with significantly lowered hemoglobin concentrations and other abnormalities. Hence, it was not possible to alter discretely the antioxidant status of RBCs without significantly perturbing other RBC parameters.

A subsequent modification of the lysis-resealing method was developed by DeLoach et al., who used dialysis membranes or hollow-core dialysis tubing to maintain the
in intracellular constituents (eg, hemoglobin, catalase) and membranes in a relatively undiluted state during lysis and resealing. However, the procedures of DeLoach et al used relatively complicated methods and equipment.39 The simplified dialysis lysis-resealing procedure we report results in efficient inclusion of small (GSH) and large (SOD) molecules into normal RBCs. These RBCs maintained relatively normal MCV, MCH, MCHC, cellular deformability, and oxidant sensitivity.

With this method, we examined the possible effect of elevated SOD activity in preventing O2·−-mediated damage. Numerous studies have implicated O2·− as a potential source of oxidative damage and as a mediator of oxidative hemolysis.14,17,40,41 Indeed, sickle cells generate substantial amounts of O2·−42 and have been reported to have reduced SOD activity.41 One might therefore anticipate that elevated SOD activity would be protective against O2·−-generating agents. However, RBCs with five- to ninefold increases in activity show no enhanced resistance to O2·− generators (PMS and menadione sodium bisulfite). This apparent inability of SOD to prevent PMS-mediated methemoglobin generation, believed to be a direct consequence of O2·− generation, has caused us to re-evaluate the importance of SOD per se in cellular oxidant defense.

In our studies, sufficient SOD was present to reduce the steady-state levels of O2·− significantly. As shown by the rate of NBT reduction in the SOD-loaded RBCs, O2·− was scavenged much more efficiently in these cells. Thus, O2·− probably is not responsible for the PMS-mediated generation of methemoglobin. In general, O2·− functions as a reductant, not as an oxidant, as would be required for generation of methemoglobin. Consequently, O2·− is more likely to reduce methemoglobin to regenerate oxyhemoglobin.44,45 Indeed, methemoglobin levels were slightly higher in SOD-loaded RBCs, indicating perhaps that O2·−-mediated reduction of methemoglobin was inhibited.

In contrast, H2O2, the dismutation product of O2·−, is an agent that produces methemoglobin.4 Hence, O2·−-generating agents may indirectly produce methemoglobin by generation of H2O2. We cannot, however, entirely rule out possible indirect effects of high SOD activity on the direct reaction between the PMS radical and hemoglobin or the loss of NADH required for the activity of methemoglobin reductase. Should either of these explanations be operative, no
differential methemoglobin generation would be expected between control and SOD-rich RBCs.

In conclusion, the resealing technique outlined in this study demonstrates that RBC antioxidant status can be effectively manipulated while normal cellular function and structure are maintained. Enhanced SOD activity did not decrease oxidant damage caused by either PMS or menadione sodium bisulfite, both potent O$_2^-$ generators. In contrast, azide (which inhibits catalase-dependent catabolism of H$_2$O$_2$) or CDNB (eliminating GSH-mediated H$_2$O$_2$ catabolism) treatment of RBCs resulted in significantly increased PMS-mediated methemoglobin generation and hemoglobin degradation in the SOD-loaded RBCs as compared with control cells. These data suggest that H$_2$O$_2$, not O$_2^-$, mediates cellular damage by O$_2^-$-generating systems. Indeed, as in other model systems, elevated SOD activity may actually enhance damage by O$_2^-$-generating systems through the accelerated formation of H$_2$O$_2$.

In light of these findings, it will be of interest to determine if increased oxidant damage occurs in erythrocytes in which elevated SOD activity or decreased H$_2$O$_2$ catabolism has been reported. Indeed, peroxidative damage is a major pathologic finding in sickle cell anemia, a disease that is also characterized by elevated intraerythrocytic O$_2^-$ generation and decreased H$_2$O$_2$ catabolizing capacity.

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