Published in final edited form as:

Free Radic Biol Med. 2010 February 15; 48(4): 519. doi:10.1016/j.freeradbiomed.2009.11.021.

The Effect of Disruption of Genes for Peroxiredoxin-2, Glutathione Peroxidase-1 and Catalase on Erythrocyte Oxidative Metabolism

Robert M. Johnson¹, Ye-Shih Ho², Dae-Yeul Yu³, Frans A. Kuypers⁴, Yaddanapudi Ravindranath⁵, and Gerard W. Goyette⁵

¹Department of Biochemistry and Molecular Biology, Wayne State School of Medicine, Detroit, MI, United States, 48201

²Institute of Environmental Health Sciences, Wayne State University, Detroit, MI, United States, 48201

³Laboratory of Human Genomics, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea, 305-806

⁴Children's Hospital of Oakland Research Institute, Oakland, CA 94609

⁵Department of Pediatrics, Wayne State School of Medicine, Detroit, MI, United States, 48201

Abstract

Peroxiredoxin-2 (Prdx2), a potent peroxide reductant, is the third most abundant protein in the erythrocyte and might be expected to play a major role in the cell's oxidative defenses. However, in this study, experiments with erythrocytes from mice with a disrupted Prdx2 gene found that the cells were not more sensitive to exogenous H₂O₂ or organic peroxides than wild-type. Intraerythrocytic H₂O₂ was increased, however, indicating an important role for Prdx2 in detoxifying endogenously-generated H₂O₂. These results are consistent with proposals that red cell Prdx2 acts stoichiometrically, not catalytically, in reducing peroxides. Additional experiments with mice with disrupted catalase or glutathione peroxidase (Gpx1) genes showed that Gpx1 is the only erythrocyte enzyme that reduces organic peroxides. Catalase(−/−) cells were readily oxidized by exogenous H₂O₂. Cells lacking both catalase and Gpx1 were more sensitive to exogenous H₂O₂ than cells lacking only catalase. A kinetic model proposed earlier to rationalize results with Gpx1(−/−) erythrocytes also fit the data with Prdx2(−/−) cells, and indicates that while Gpx1 and Prdx2 both participate in removing endogenous H₂O₂, Prdx2 plays a larger role. Although the rate of H₂O₂ production in the red cell is quite low, Prdx2-deficient mice are anemic, suggesting an important role in erythropoiesis.

Keywords

peroxiredoxin; erythrocyte; catalase; glutathione peroxidase; oxidation

© 2009 Elsevier Inc. All rights reserved.

Corresponding author: Dr. Robert M. Johnson Department of Biochemistry and Molecular Biology Wayne State School of Medicine 540 E. Canfield Detroit, MI 48201 rmjohns@med.wayne.edu Tel: 313-577-1816.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Since 1-2% of the cell's oxyhemoglobin undergoes spontaneous heterolytic dissociation into metHb and superoxide every day [1], and superoxide is readily converted to H_2O_2 by superoxide dismutase, the red cell is unavoidably exposed to these reactive oxidizing species (ROS). The survival of the erythrocyte therefore depends on an efficient defense against oxidative damage by H_2O_2 and superoxide. Three enzymes are believed to participate in the oxidative defense of the red cell: glutathione peroxidase-1 (Gpx1), catalase, and peroxiredoxin-2 (Prdx2). We have begun to examine the function of each of these enzymes using a series of mice in which the gene for one or more of these enzymes has been disrupted. Experiments [2] with the catalase inhibitor 3-amino-1,2,4-triazole (3-AT) found that very little endogenously produced H_2O_2 is catabolized by catalase in wild-type mouse red cells, but that deletion of Gpx1 increased H_2O_2 flux through catalase, indicating that Gpx1 plays a major role in eliminating endogenous H_2O_2 . In addition, we were able to show that Gpx1 protects the erythrocyte against attack by organic peroxides [3], as proposed many years ago by Flohé [4]. These experiments with Gpx1 deficient cells, together with a detailed kinetic model of the cell's oxidative metabolism [2], found that the activity of catalase and glutathione peroxidase are inadequate to explain the oxidative catabolism of the red cell, and that peroxiredoxin activity had to be included in the model to explain the experimental results.

We here report experiments testing this proposal. The major peroxiredoxin of the mature red cell is Prdx2 [5]. Mice with a disrupted Prdx2 gene have been constructed [6]. These animals are anemic and show various red cell abnormalities. We found that deletion of Prdx2 led to an increase in erythrocyte H_2O_2 , confirming a role for Prdx2 in the cell's endogenous H_2O_2 metabolism. The loss of Prdx2 had no effect on the ability of the mature red cell to detoxify exogenous H_2O_2 . In addition, although Prdx2 is able to reduce organic peroxides *in vitro*, it appears not to do this in the red cell. Rather, Gpx1 has the primary role in detoxifying organic peroxides. The data also confirm that exogenous H_2O_2 is detoxified primarily by catalase.

The presence of high levels of Prdx2 together with the high rate of reaction between Prdx2 and H_2O_2 has led to proposals that Prdx2 is a major antioxidant protein in the red cell [6,7]. Our data suggest that erythrocytic Prdx2 participates in the disposal of endogenous H_2O_2 , but plays no role in eliminating exogenous peroxides. This relatively minor role for Prdx2 in the mature red cell, despite its high concentration, lends support to the proposal [5] that it has its greatest importance in hematopoiesis.

Methods

Knockout mice

The generation of mice deficient in each of the following enzymes has been described: Gpx1 [8]; and catalase [9]. The Gpx1 and catalase double knockout mice were generated by intercrossing of Gpx1 knockout mice and catalase knockout mice that had been backcrossed to C57BL/6 mice for 8 and 10 generations, respectively. The Prdx2(−/−) mice, which were constructed by Lee et al [6] and had been backcrossed to C57BL/6 mice, were obtained from a stock maintained at the Children's Hospital of Oakland Research Institute, Oakland, CA. As controls, age-matched wild-type C57BL/6 mice were used.

Erythrocytes

Blood was obtained from the hearts of enzyme-deficient mice and the C57BL/6 control mice after pentobarbital anesthesia or CO_2 asphyxiation. The erythrocytes were washed twice with PBS (145 mM NaCl, 5 mM NaPi, 1 mM EDTA, pH 7.4). In most cases, white cells were removed by filtration through cellulose [10]. For the incubations, the cells were then resuspended three times in Krebs-Ringer (KR) buffer (143 mM NaCl, 5.7 mM KCl, 1.4 mM

MgCl₂, 18 mM sodium phosphate, pH 7.4, with 10 mM glucose and 50 mg/ml gentamycin added immediately before use).

Enzyme assays

Catalase was assayed by the method of Chance [11], as modified by Aebi [12]. 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 H₂O₂ in buffer. Readings were obtained at 15 and 30 sec, and k, the first order rate constant, was calculated. Grieshaber and Hoffman [13] reported that tetramer of C57BL/6 catalase has a k' of $2.5 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, and this value was used to estimate the catalase concentration (k/k'). 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 [14].

The Prdx2(−/−) erythrocytes are hypocatalasemic relative to the C57BL/6 background strain, with about 80% of the catalytic activity of the C57BL wild-type. Since catalase has not been purified from the Prdx2(−/−) erythrocytes, it is not known whether this is due to underproduction or a mutated enzyme. For the purposes of this paper, we have assumed underproduction.

Catalase inhibition by 3-AT

The reaction mechanism of catalase has two steps [15]. In the first, catalase transfers two electrons to H₂O₂, 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 H₂O₂, restoring the ground state:

Catalase Compound I is specifically and irreversibly inhibited by covalent reaction with 3-amino-2,4,5-triazole (3-AT) [16]. Thus, the rate of catalase inactivation by 3-AT should be related to H₂O₂ levels.

To do the experiments, red cells were washed three times in Krebs-Ringer buffer and resuspended in Krebs-Ringer. Incubation mixtures containing 0.5 ml of the cell suspension with either 10 μ l of 2.5 M 3-AT in water (50 mM final concentration) or 10 μ l of water (control) were incubated at 37°. At intervals, 50 μ l samples were removed and quickly centrifuged. The supernatant was discarded and the cells were washed twice in cold Krebs-Ringer buffer. The cell pellet was lysed by the addition of 1.0 ml of cold water. 100 μ l were taken for catalase assay, and 400 μ l for hemoglobin assay with Drabkin's reagent. 3-AT was recrystallized from ethanol before use. We found that commercial 3-AT has an impurity which inactivates ground state catalase.

Other assays

H₂O₂ was assayed by the method of Green and Hill [17]. The concentration of H₂O₂ in the stock solution was quantitated [18] using an A₂₄₀ value of 43.6 M^{−1}cm^{−1}. 3-AT was assayed by the method of Agrawal and Margoliash [19]. GSH was determined with Ellman's reagent as described earlier [20]. Methemoglobin (methHb) was determined by the Evelyn and Molloy

method [21] or spectroscopically [22]. For spectroscopy, the simultaneous equation coefficients were recalculated using the absorbance values of Zijlstra et al [23] for HbO₂, metHb and Hb.

Unless otherwise noted, reagents were obtained from Sigma.

Results

The effect of exogenous H₂O₂ on catalase-deficient, Gpx1-deficient, and double knockout red cells

Catalase is generally believed to be the major red cell defense against exogenous H₂O₂. Consistent with this, hemoglobin in catalase(−/−) red cells was readily oxidized by H₂O₂ (figure 1). Catalase(+/−) cells were as resistant to H₂O₂ challenge as wild-type erythrocytes. This is consistent with clinical data on individuals with acatalasemia, whose red cells have low but not zero catalase activity and are phenotypically normal [24]. When homozygous catalase (−/−) mouse red cells were exposed to a range of H₂O₂ fluxes generated by glucose oxidase, extensive Hb oxidation was observed (figure 2). As noted earlier [2,25], Gpx1(−/−) red cells, with normal levels of catalase, were not oxidized by the addition of H₂O₂. In contrast, cells deficient in both catalase and Gpx1 were more sensitive to exogenous H₂O₂ than cells with catalase deficiency alone (figure 2). Thus, while catalase is the major defense against exogenous H₂O₂, Gpx1 also participates in this function. Similar results were obtained when cells were challenged by a single bolus of H₂O₂ (figure 3).

Catalase-deficient red cells and organic peroxides

Catalase is highly specific for H₂O₂ as a substrate and is not expected to detoxify organic peroxides. Consistent with this expectation, catalase deficient red cells had no increased sensitivity to oxidation by either *tert*-butyl hydroperoxide or cumene hydroperoxide (figure 4). Gpx1(−/−) red cells are oxidized at a greater rate by organic peroxides than are wild-type [3]. This finding is verified here, and adding catalase deficiency to Gpx1 deficiency did not increase Hb oxidation by organic peroxide (data not shown). These results are consistent with the expectation that catalase plays no part in organic peroxide detoxification.

Prdx2(−/−) red cells and exogenous H₂O₂

Prdx2 reacts very rapidly with H₂O₂ [26-28], suggesting that Prdx2 may play a role in protecting erythrocytes against oxidant challenge. If this were so, Prdx2(−/−) red cells might be especially susceptible to H₂O₂ exposure. However, red cells without Prdx2 did not show increased susceptibility to exogenous H₂O₂. Prdx2(−/−) red cells did not differ from wt cells when treated with bolus additions of H₂O₂ (figure 5). This result is consistent with the observation of Peskin et al [28] that exogenous H₂O₂, even at micromolar levels, converts red cell Prdx2 to an inactive disulfide linked dimer. This dimer is converted back to the active form very slowly, because red cell thioredoxin levels are low.

Are Prdx2(−/−) red cells more sensitive to organic peroxides?

It has been shown that a primary function of Gpx1 in red cells is defense against organic peroxides [3,4]. Prdx2 is able to reduce organic peroxides about as well as H₂O₂ [28], and thus might supplement the primary role of Gpx1. However, exposure to a range of concentrations of cumene hydroperoxide or *tert*-butyl hydroperoxide led to equivalent levels of MetHb formation in both wt and Prdx2(−/−) red cells (figure 6), showing that Prdx2 plays a negligible role in detoxifying organic peroxides in the red cell despite its high reactivity *in vitro*.

Prdx2(−/−) red cells and endogenous H₂O₂

If Prdx2 is a factor in the removal of endogenous H₂O₂, as proposed previously [2], it is expected that concentrations of endogenously generated H₂O₂ will be higher in Prdx2(−/−) cells than in wild-type. 3-AT irreversibly inhibits the Compound I intermediate of catalase [16,29], which forms only if catalase is actively reducing H₂O₂. Thus, the rate of irreversible catalase inhibition by 3-AT in the red cell is a monitor of catalase activity, which will increase as endogenous H₂O₂ levels rise. We therefore determined if 3-AT treatment led to a more rapid loss of catalase activity in Prdx2(−/−) red cells than in wild-type. In the experiment, red cells from wt and knockout mice were incubated with 50 mM 3-AT and residual catalase activity was assayed for 3 hours.

Figure 7 shows the result of a representative experiment. In wild-type mouse red cells, as was noted earlier [2], there is a slow loss of catalase activity with 3-AT, with a rate of 23 nM/h. Notably, a higher rate of catalase inactivation, 37 nM/h, was observed in Prdx2(−/−) cells, indicating an increased H₂O₂ level in Prdx2(−/−) cells, compared to wt. Thus, Prdx2 plays a role in regulating endogenous H₂O₂ levels.

Modeling H₂O₂ metabolism in Prdx2(−/−) red cells

The finding that catalase is inhibited by 3-AT in Prdx2-deficient red cells is consistent with a simple model of oxidative reactions in the erythrocyte [2]. This model comprises 15 reactions, which generate seven simultaneous differential equations (Supplemental Materials). In this work, one rate constant has been modified: for Gpx1, the first-order rate constant of Mueller et al [30] used previously [2] has been replaced by the more physiological Michaelis-Menten rate equation [31]. It is known that the kinetic mechanism of Gpx1 is ter uni [32], but the measurements of Flohé et al with purified enzyme demonstrated that no ternary enzyme-substrate complexes are formed during catalysis. Consequently, Gpx1 kinetics are in fact Michaelis-Menten, as Paglia and Valentine observed [31]. The model predicts [2] that Prdx2 would play a role in regulating erythrocyte H₂O₂, which is verified here (figure 7). The model's predicted rates of catalase inactivation in the presence of 3-AT, as shown as dashed lines in figure 7, are in excellent agreement with the experimental results. The solid lines in figure 7 show the least squares fits of the data.

How much endogenous H₂O₂ does each enzyme remove?

The previous 3-AT inhibition results for cells with gene deletions for Gpx1 [2] is also well modeled using Paglia and Valentine kinetics (data not shown). To estimate the relative importance of Prdx2 and Gpx1 in reducing the red cell's endogenous H₂O₂, Table 1 shows the calculated level of H₂O₂ in wild-type and various knockout red cells. The absence of Prdx2 increases intracellular H₂O₂ by 400% to 10.1×10^{-10} M, while the absence of Gpx1 increases intracellular H₂O₂ by only 20%, to 3.2×10^{-10} M. Thus, Prdx2 appears to be the more important enzyme for detoxifying endogenously generated H₂O₂. The model predicts that the fraction of catalase as Compound I is increased from 6% in wt to 14% in the Prdx(−/−) cells. Since Compound I is the form of catalase that is inhibited by 3-AT, the rate of irreversible catalase inhibition is predicted to be about twice as high in Prdx2(−/−) red cells than wild-type, as is observed (figure 7).

Catalase activity is negligible in these conditions. The first order rate for catalase in the wt red cell is about $5 \times 10^{-7} \text{ M} \times 1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1} = 5/\text{s}$, where $5 \times 10^{-7} \text{ M}$ is the catalase concentration in the cell and $1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ is the estimated second order rate constant [15].

Oxidative damage in knockout cells

Biochemical data for some oxidation relevant components are given in Table 2. Most values are normal, but catalase ($-/-$) cells have evidence of oxidative damage incurred in the circulation: membrane thiols are significantly oxidized. In double knockouts [catalase($-/-$) plus Gpx1($-/-$)], %metHb is also slightly elevated.

Discussion

These results confirm some earlier proposals and offer new insights into oxidative defense in the red cell.

We earlier proposed [2] that Prdx2 would play a role in protecting the red cells from oxidative damage by its endogenously generated H_2O_2 . The availability of erythrocytes from Prdx2($-/-$) mice allowed a critical test of this hypothesis. The oxidative defenses of Prdx2($-/-$) red cells were tested in three ways, by asking (a) if endogenous H_2O_2 levels are elevated enough in Prdx2($-/-$) red cells to make catalase a major scavenger of H_2O_2 ; (b) whether the cell's ability to resist exogenous H_2O_2 is affected; (c) whether resistance to organic peroxides is affected.

Endogenous H_2O_2 was assessed by determining the rate of irreversible catalase inhibition by 3-AT. In Prdx2($-/-$) cells, there was an increase in catalase inactivation relative to wt, indicating increased endogenous H_2O_2 levels in these cells. This is in accord with earlier modeling [2] and is consistent with the conclusions of Low et al [7] that Prdx2 acts a stoichiometric scavenger of H_2O_2 in the red cell.

Perhaps surprisingly, Prdx2 appeared to play no role in eliminating exogenous H_2O_2 in the red cell, despite its ability to react with H_2O_2 at a high rate [26-28] and its abundance in the red cell [33]. However, this finding is consistent with the observation of Low et al [7] that erythrocyte Prdx2 is oxidized to an inactive disulfide-linked dimer by H_2O_2 concentrations as low as 1 μ M, and that this species is reduced to the active form very slowly, because of a low erythrocytic level of thioredoxin reductase. In our experiments with exogenous H_2O_2 at μ M levels, Prdx2 in wt cells will be rapidly inactivated and play no role in reducing H_2O_2 . Thus, the presence or absence of Prdx2 will not affect the response to exogenous H_2O_2 . This is in contrast with the role of Prdx2 in removing endogenous H_2O_2 . The rate of endogenous production is so low that the rate of formation of the Prdx2 dimer and its reduction to the active sulfhydryl form are comparable, and Prdx2 is able to participate in reducing endogenous H_2O_2 .

In contrast with the results reported here, Lee et al [6] observed that Prdx2($-/-$) red cells were more sensitive to H_2O_2 challenge than wt cells. This difference may be a consequence of the use of different background strains. Our Prdx2($-/-$) mice were the C57BL/6 strain, whereas Lee et al used 129SV/J. Epistasis is often observed in knockout mouse strains (reviewed in [34,35]) and it is likely that the effects of a Prdx2 deletion may vary in different genetic backgrounds.

Similarly, deletion of Prdx2 had no effect of the cell's ability to eliminate organic peroxides, even though Prdx2 *in vitro* is a potent peroxidase for organic peroxides [28,36]. This may also be a consequence of the inability to regenerate reduced Prdx2 after one round of catalysis. This hypothesis requires additional experimentation.

There has been a longstanding controversy about the relative importance of Gpx1 and catalase in protecting the red cell from damage by H_2O_2 [30,37-43] (reviewed in [5]). The results reported here and earlier show, however, that it is an oversimplification to assign any one

enzyme as the route of H_2O_2 detoxification. All three H_2O_2 catabolizing enzymes contribute and their relative importance depends on the ambient peroxide level. For example, Mueller et al [30] concluded that catalase was the most significant enzyme, but their experiments were conducted with $1 \mu\text{M}$ H_2O_2 , well above the probable intraerythrocytic H_2O_2 concentration of 0.05 nM (table 1). At an endogenous H_2O_2 concentration of 0.05 nM , the significant defense enzymes are Gpx1, as proposed many years ago by Hochstein's group [37], and Prdx2 (figure 7).

Although the contribution of catalase to the control of endogenous H_2O_2 is negligible, the major role of catalase [30,40,43] in detoxifying exogenous H_2O_2 is confirmed. Disruption of the catalase gene yields red cells that are highly susceptible to oxidation by exogenous H_2O_2 . Catalase is also important to one of the red cell's *in vivo* functions, since red cells are known to be sinks for H_2O_2 in the circulation [44], Catalase(−/−) cells in the circulation appear to be unable to carry out this function effectively and are in fact damaged by the H_2O_2 that they take up, since membrane thiols are diminished, presumably by oxidation.

We have proposed [2] that both catalase and Gpx1 participate in detoxification of high levels of exogenous H_2O_2 . This proposal finds further support in the observation that red cells deficient in both catalase and Gpx1 are more readily oxidized than cells lacking catalase alone.

Previously, a simplified model of red cell oxidation and defense [2] predicted that the inclusion of Prdx2 activity would be necessary to explain the experimental results with 3-AT. With the replacement of a first-order rate constant for Gpx1 with Michaelis-Menten kinetics, the model fits the results reported here (figure 7), as well as the earlier data. The success of the model depends on its restriction to endogenous H_2O_2 concentrations. Predicting the response to high exogenous levels of H_2O_2 will require accurate rate constants for Prdx2 oxidation and reversal and the inclusion of higher Hb oxidation states.

One element of the experimental results is not predicted by the kinetic modeling, i.e., the massive increase in metHb seen in catalase deficient red cells in response to a bolus addition of H_2O_2 . The model predicts a transient increase in metHb with a subsequent return to normal levels as exogenous H_2O_2 is eliminated by catalase. This is not observed. Although the added H_2O_2 is rapidly reduced to undetectable levels, metHb, rather than declining, continues to rise. This suggests that the exogenous H_2O_2 has led to the formation of unstable non-reducible Hb species. Such species have been observed in normal and acatalasemic red cells exposed to H_2O_2 [45-47]. Extensive oxidative damage can be produced when Hb is denatured sufficiently to release Fe^{2+} , a potent redox reagent.

It is often said that red cells are exposed to an especially high level of oxidative stress because of their high content of oxygenated Hb (ranging from 3 to 5 mM). However, if the only source of erythrocyte ROS is the autooxidation of 2-3% of its oxyHb per day, the rate of superoxide formation is about 2 nM/s . This represents the upper limit for the rate of H_2O_2 production, since some of the superoxide recombines with metHb to regenerate oxyHb [48]. This rate is in fact quite low compared to the rate of H_2O_2 generation by actively respiring cells. In bacteria, where this number can be accurately determined [49], the rate of H_2O_2 production is $14 \mu\text{M/s}$, 700 times greater than endogenous red cell production. Thus, endogenous oxidative stress in the red cell is very small and is readily handled by Gpx1 and Prdx2.

A puzzle remains in the observation that red cells from Prdx2(−/−) mice are damaged and the mice are anemic [6]. Since Prdx2 is not essential in protecting the red cell from endogenously generated H_2O_2 , yet Prdx2(−/−) mice have anemia, it is tempting to suggest [5] that Prdx2 plays its primary role in erythropoiesis rather than in maintenance of the mature red cell. Support for this lies in the dual function of peroxiredoxin, as an antioxidant enzyme and as a chaperone [50]. We propose that the chaperone function is especially important in the stages

of erythropoiesis that see high rates of Hb synthesis. Failure to properly fold globin into hemoglobin will result in unsequestered heme and Fe²⁺ in the nascent cells, with the attendant promotion of oxidative reactions.

A related puzzling feature of red cell Prdx2 is its high concentration in the mature erythrocyte, despite its relatively limited anti-oxidant role. Here as well, the chaperone function of Prdx2 may be its more important activity. Recombinant Prdx can prevent methemoglobin formation in red cell hemolysates [51], and Prdx2 may act to limit or reverse hemoglobin denaturation during the cell's time in the circulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH Grant HL56421 (Y-S. H.) and the Ginopolis Fund of Children's Hospital of Michigan.

References

1. Hsieh, H-S.; Jaffé, E. The metabolism of methemoglobin in human erythrocytes. In: Surgenor, DM., editor. *The Red Blood Cell*. Academic Press; New York City: 1975. p. 799-824.
2. Johnson RM, Goyette G Jr, Ravindranath Y, Ho YS. Hemoglobin autoxidation and regulation of endogenous H₂O₂ levels in erythrocytes. *Free Radic Biol Med* 2005;39:1407–17. [PubMed: 16274876]
3. Johnson RM, Goyette G Jr, Ravindranath Y, Ho YS. Oxidation of glutathione peroxidase-deficient red cells by organic peroxides. *Blood* 2002;100:1515–6. [PubMed: 12184279]
4. Flohé L. Glutathione peroxidase: fact and fiction. *Ciba Found Symp* 1978;65:95–122. [PubMed: 383423]
5. Low FM, Hampton MB, Winterbourn CC. Peroxiredoxin 2 and peroxide metabolism in the erythrocyte. *Antioxid Redox Signal* 2008;10:1621–30. [PubMed: 18479207]
6. Lee TH, Kim SU, Yu SL, Kim SH, Park DS, Moon HB, Dho SH, Kwon KS, Kwon HJ, Han YH, Jeong S, Kang SW, Shin HS, Lee KK, Rhee SG, Yu DY. Peroxiredoxin II is essential for sustaining life span of erythrocytes in mice. *Blood* 2003;101:5033–8. [PubMed: 12586629]
7. Low FM, Hampton MB, Peskin AV, Winterbourn CC. Peroxiredoxin 2 functions as a noncatalytic scavenger of low-level hydrogen peroxide in the erythrocyte. *Blood* 2007;109:2611–7. [PubMed: 17105810]
8. Ho Y, Magnenat J, Bronson R, Cao J, Gargano M, Sugawara M, Funk C. Mice deficient in cellular glutathione peroxidase develop normally and show no increased sensitivity to hyperoxia. *J Biol Chem* 1997;272:16644–16651. [PubMed: 9195979]
9. Ho Y, Xiong Y, Ma W, Spector A, Ho D. Mice lacking catalase develop normally but show differential sensitivity to oxidant tissue injury. *J Biol Chem* 2004;279:32804–32812. [PubMed: 15178682]
10. Beutler E, West C, Blume KG. The removal of leukocytes and platelets from whole blood. *J Lab Clin Med* 1976;88:328–33. [PubMed: 956688]
11. Chance B. Catalases and Peroxidases. II. Special methods. *Meth Biochem Anal* 1954;1:408–424.
12. Aebi H. Catalase in vitro. *Meth. Enz* 1984;105:121–126.
13. Grieshaber C, Hoffman H. Genetic regulation of the expression of catalase activity in murine red blood cells. *Biochem Genet* 1975;13:447–456. [PubMed: 810135]
14. Foster, H.; Small, J.; Fox, J. *The mouse in biomedical research*. Academic Press; New York: 1983. p. 302III
15. Nicholls, P.; Fita, I.; Loewen, PC. Enzymology and structure of catalases. In: Sykes, A.; Mauk, G., editors. *Heme-Fe Proteins: Advances in Inorganic Chemistry*. Academic Press; San Diego, CA: 2001. p. 51-106.51 51

16. Margoliash E, Novogrodsky A, Schejter A. Irreversible reaction of 3-amino-1:2:4-triazole and related inhibitors with the protein of catalase. *Biochem J* 1960;74:339–350. [PubMed: 13848609]
17. Green M, Hill H. Chemistry of dioxygen. *Methods Enzymol* 1984;105:3–22. [PubMed: 6328186]
18. Claiborne, A. Catalase activity. In: Greenwald, R., editor. *CRC Handbook of Methods for Oxygen Radical Research*. CRC Press; Boca Raton, Florida: 1985. p. 283-284.
19. Agrawal B, Margoliash E. A spectrophotometric method for the determination of aminotriazole and other aromatic amines. *Anal Biochem* 1970;34
20. Johnson RM, Ravindranath Y, el-Alfy M, Goyette G Jr. Oxidant damage to erythrocyte membrane in glucose-6-phosphate dehydrogenase deficiency: correlation with in vivo reduced glutathione concentration and membrane protein oxidation. *Blood* 1994;83:1117–1123. [PubMed: 8111051]
21. Dacie, J.; Lewis, S. *Practical Hematology*. 7th ed. Churchill Livingstone; Edinburgh: 1991. p. 556
22. Winterbourn C. Oxidative reactions of hemoglobin. *Meth. Enz* 1990;186:265–272.
23. Zijlstra WG, Buursma A, Meeuwse-van der Roest WP. Absorption spectra of human fetal and adult oxyhemoglobin, de-oxyhemoglobin, carboxyhemoglobin, and methemoglobin. *Clin Chem* 1991;37:1633–8. [PubMed: 1716537]
24. Ogata M, Mizugaki J. Properties of erythrocyte catalase from heterozygotes for Japanese type acatalasemia. *Acta Med Okayama* 1979;33:205–11. [PubMed: 40400]
25. Johnson R, Goyette G Jr, Ravindranath Y, Ho Y-S. Red cells from glutathione peroxidase-1-deficient mice have nearly normal defenses against exogenous peroxides. *Blood* 2000;96:1985–1988. [PubMed: 10961904]
26. Manta B, Hugo M, Ortiz C, Ferrer-Sueta G, Trujillo M, Denicola A. The peroxidase and peroxynitrite reductase activity of human erythrocyte peroxiredoxin 2. *Arch Biochem Biophys* 2009;484:146–154. [PubMed: 19061854]
27. Ogusucu R, Rettori D, Munhoz DC, Netto LE, Augusto O. Reactions of yeast thioredoxin peroxidases I and II with hydrogen peroxide and peroxynitrite: rate constants by competitive kinetics. *Free Radic Biol Med* 2007;42:326–34. [PubMed: 17210445]
28. Peskin AV, Low FM, Paton LN, Maghzal GJ, Hampton MB, Winterbourn CC. The high reactivity of peroxiredoxin 2 with H₂O₂ is not reflected in its reaction with other oxidants and thiol reagents. *J Biol Chem* 2007;282:11885–92. [PubMed: 17329258]
29. Margoliash, E.; Schejter, A. Irreversible inhibition of catalase by the 3-amino-1:2:4-triazole group of inhibitors in the presence of catalase donors. In: Falk, J.; Lemberg, R.; Morton, R., editors. *Hematin Enzymes*. Pergamon Press; Oxford: 1961. p. 236-244.1 1
30. Mueller S, Riedel HD, Stremmel W. Direct evidence for catalase as the predominant H₂O₂-removing enzyme in human erythrocytes. *Blood* 1997;90:4973–8. [PubMed: 9389716]
31. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med* 1967;70:158–69. [PubMed: 6066618]
32. Flohé L, Loschen G, Gunzler WA, Eichele E. Glutathione peroxidase, V. The kinetic mechanism. *Hoppe Seylers Z Physiol Chem* 1972;353:987–99. [PubMed: 5066111]
33. Moore R, Mankad M, Shriver S, Mankad V, Plishker G. Reconstitution of Ca(2+)-dependent K⁺ transport in erythrocyte membrane vesicles requires a cytoplasmic protein. *J Biol Chem* 1991;266:18964–8. [PubMed: 1918011]
34. Nadeau JH. Modifier genes in mice and humans. *Nat Rev Genet* 2001;2:165–74. [PubMed: 11256068]
35. Phillips PC. Epistasis--the essential role of gene interactions in the structure and evolution of genetic systems. *Nat Rev Genet* 2008;9:855–67. [PubMed: 18852697]
36. Cordray P, Doyle K, Edes K, Moos PJ, Fitzpatrick FA. Oxidation of 2-Cys-peroxiredoxins by arachidonic acid peroxide metabolites of lipoxygenases and cyclooxygenase-2. *J Biol Chem* 2007;282:32623–9. [PubMed: 17855346]
37. Cohen G, Hochstein P. Glutathione peroxidase: the primary agent for the elimination of hydrogen peroxide in erythrocytes. *Biochem* 1963;2:1420–1428. [PubMed: 14093920]
38. Gaetani GF, Ferraris AM, Rolfo M, Mangerini R, Arena S, Kirkman HN. Predominant role of catalase in the disposal of hydrogen peroxide within human erythrocytes. *Blood* 1996;87:1595–1599. [PubMed: 8608252]

39. Gaetani GF, Galiano S, Canepa L, Ferraris AM, Kirkman HN. Catalase and glutathione peroxidase are equally active in detoxification of hydrogen peroxide in human erythrocytes. *Blood* 1989;73:334–9. [PubMed: 2491951]
40. Gaetani GF, Kirkman HN, Mangerini R, Ferraris AM. Importance of catalase in the disposal of hydrogen peroxide within human erythrocytes. *Blood* 1994;84:325–330. [PubMed: 8018928]
41. Mills G. Hemoglobin catabolism. I. Glutathione peroxidase, an erythrocyte enzyme which protects hemoglobin from oxidative breakdown. *J Biol Chem* 1957;229:189. [PubMed: 13491573]
42. Nicholls P. Contributions of catalase and glutathione peroxidase to red cell peroxide removal. *Biochim Biophys Acta* 1972;279:306–309. [PubMed: 5082500]
43. Scott MD, Lubin BH, Zuo L, Kuypers FA. Erythrocyte defense against hydrogen peroxide: preeminent importance of catalase. *J Lab Clin Med* 1991;118:7–16. [PubMed: 2066646]
44. Winterbourn CC, Stern A. Human red cells scavenge extracellular hydrogen peroxide and inhibit formation of hypochlorous acid and hydroxyl radical. *J Clin Invest* 1987;80:1486–91. [PubMed: 2824562]
45. Masuoka N, Wakimoto M, Ohta J, Ishii K, Nakano T. Characterization of hydrogen peroxide removal activities in mouse hemolysates: catalase activity and hydrogen peroxide removal activity by hemoglobin. *Biochim Biophys Acta* 1997;1361:131–7. [PubMed: 9300794]
46. Vollaard NB, Reeder BJ, Shearman JP, Menu P, Wilson MT, Cooper CE. A new sensitive assay reveals that hemoglobin is oxidatively modified in vivo. *Free Radic Biol Med* 2005;39:1216–28. [PubMed: 16214037]
47. Balagopalakrishna C, Manoharan PT, Abugo OO, Rifkind JM. Production of superoxide from hemoglobin-bound oxygen under hypoxic conditions. *Biochem* 1996;35:6393–8. [PubMed: 8639585]
48. Winterbourn CC, McGrath BM, Carrell RW. Reactions involving superoxide and normal and unstable haemoglobins. *Biochem J* 1976;155:493–502. [PubMed: 182128]
49. Seaver LC, Imlay JA. Hydrogen peroxide fluxes and compartmentalization inside growing *Escherichia coli*. *J Bacteriol* 2001;183:7182–9. [PubMed: 11717277]
50. Jang HH, Lee KO, Chi YH, Jung BG, Park SK, Park JH, Lee JR, Lee SS, Moon JC, Yun JW, Choi YO, Kim WY, Kang JS, Cheong GW, Yun DJ, Rhee SG, Cho MJ, Lee SY. Two enzymes in one; two yeast peroxiredoxins display oxidative stress-dependent switching from a peroxidase to a molecular chaperone function. *Cell* 2004;117:625–35. [PubMed: 15163410]
51. Stuhlmeier KM, Kao JJ, Wallbrandt P, Lindberg M, Hammarstrom B, Broell H, Paigen B. Antioxidant protein 2 prevents methemoglobin formation in erythrocyte hemolysates. *Eur J Biochem* 2003;270:334–41. [PubMed: 12605684]
52. Macey, R.; Oster, G. Madonna, 8.3.18. Kagi; Berkeley, CA: 2006.

Figure 1.

Absence of catalase makes red cell hemoglobin susceptible to oxidation by H₂O₂. To red cells from catalase deficient (-/-), heterozygotes (-/+), and wild-type mice at a hematocrit of 5 in PBS containing 10 mM glucose were added appropriate amounts of 1 mM H₂O₂ and buffer to achieve the indicated concentrations. MetHb was determined after 15 minutes at room temperature.

Figure 2.

Hb oxidation in enzyme-deficient red cells. In this experiment, washed cellulose-filtered red cells from wild-type mice (triangles) or from mice with disrupted genes for catalase (open squares), Gpx1 (filled circles), or both (open circles) were suspended in KR buffer (Hb = 30 mg/ml) at 37°. Glucose oxidase was added (1mU/ml) to generate 0.137 μM H_2O_2 /min. MetHb was assayed at 0, 2, 4, 6 hours. Two runs are shown here to indicate the usual level of agreement between duplicate runs. Subsequent figures will present averages of two or more duplicate runs. Double KO red cells were more sensitive to an oxidative challenge than cells deficient in catalase alone.

Figure 3.

Hb oxidation in enzyme-deficient red cells in response to bolus additions of H_2O_2 . Washed red cells in KR buffer (Hct = 5) at 37° were mixed with 30 mM H_2O_2 and buffer to achieve the indicated $[\text{H}_2\text{O}_2]$. MetHb was determined after 15 minutes at 37° . Symbols as in figure 2.

Figure 4.

Catalase(-/-) red cells are not sensitive to organic peroxides. Duplicate runs of washed red cells at a hematocrit of 5 were incubated in KR buffer with 10 mM glucose and different amounts of *tert*-butylhydroperoxide (top) or cumene hydroperoxide (bottom) at 37°. MetHb was assayed at the end of 1 hour.

Figure 5.

Prdx2(-/-) red cells are not more sensitive to oxidation by exogenous H₂O₂. Washed cellulose-filtered red cells in KR buffer (Hct = 5) at 37° were mixed with 30 mM H₂O₂ and buffer to achieve the indicated [H₂O₂]. MetHb was determined after 15 minutes at 37°. (Patterned bars are wt; filled bars are Prdx(-/-) cells.)

Figure 6.

Prdx2(-/-) red cells do not have increased sensitivity to organic peroxides. Wild-type red cells (open circles) and Prdx2 red cells (filled circles) at a hematocrit of 5 were incubated in KR buffer with 10 mM glucose and different amounts of (a) cumene hydroperoxide or (b) *tert* - butylhydroperoxide at 37°. MetHb was assayed at the end of 1 hour.

Figure 7.

Time course of catalase inhibition by 50 mM 3-AT in Prdx2(-/-) red cells (filled circles) and wt cells (open circles). The rate of catalase inactivation in wt is 23 nM/h and 37 nM/h in Prdx2 (-/-) cells. (- - -) catalase activities in the presence of 3-AT predicted by the model. (—) least squares fit of the data.

Table 1

Calculated levels of superoxide, H₂O₂ and catalase in wild-type and knockout red cells. The tetramer concentrations are experimental values. The remaining entries are steady state values calculated by running the model for 250 h, using the Chemical Reactions module of Berkeley Madonna [52].

	Wt	Prdx2(-/-)	Gpx1(-/-)	catalase(-/-)	both catalase(-/-) and Gpx1(-/-)
O ₂ ⁻ (M)	5.2×10 ⁻¹³	5.2×10 ⁻¹³	5.2×10 ⁻¹³	5.2×10 ⁻¹³	5.2×10 ⁻¹³
H ₂ O ₂ (M)	2.6×10 ⁻¹⁰	10.1×10 ⁻¹⁰	3.2×10 ⁻¹⁰	2.7×10 ⁻¹⁰	3.3×10 ⁻¹⁰
Catalase [tetramer]	5.8×10 ⁻⁷	4.6×10 ⁻⁷	5.8×10 ⁻⁷	0	0
Ground state catalase (M)	5.46×10 ⁻⁷	3.94×10 ⁻⁷	5.4×10 ⁻⁷	0	0
Compound I (M)	0.35×10 ⁻⁷	0.66×10 ⁻⁷	0.41×10 ⁻⁷	0	0

Table 2

Biochemical analysis of enzyme-deficient red cells

	Thiols:	GSH	metHb
	nmol/mg membrane protein	μmol/gHb	%
Wild-type	57.7 ± 2.0 (8)	6.72 ± 0.37 (9)	1.54 ± 0.37 (22)
Catalase (+/-)	56.4 ± 0.78 (2)	7.80 ± 0.24 (4) [†]	1.35 ± 0.37 (4)
Catalase (-/-)	47.8 ± 1.25 (3) [†]	6.57 ± 0.46 (3)	1.52 ± 0.34 (17)
Catalase (-/-) + Gpx1 (-/-)	45.1 ± 2.34 (3) [†]	6.24 ± 0.19 (3)	2.03 ± 0.44 (10) [†]
Prdx2 (-/-)	55.6 ± 1.88 (3)	7.09 ± 0.58 (3)	1.52 ± 1.05 (3)

(n) indicates the number of individual blood samples assayed

[†] different from wild-type with p<0.001