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Chapter

Interplay between Erythrocyte Peroxidases and Membrane

Daniela Melo, Susana Rocha, Susana Coimbra and Alice Santos Silva

Abstract

Red blood cells (RBCs) are continuously exposed to oxidative stress (OS), mainly due to their primary function as oxygen carriers. Since RBC is a unique cell, without nucleus or other organelles, it presents a very special metabolism and a highly efficient antioxidant system to face OS conditions. Hemoglobin and RBC membrane are the major targets of oxidative modifications when RBC antioxidant capacity is overwhelmed. Fortunately, non-enzymatic agents, such as glutathione, and enzymatic agents, namely, several peroxidases, such as catalase, glutathione peroxidase and peroxiredoxin 2, are able to prevent OS damage. Although these peroxidases are mainly cytosolic enzymes, evidence exists about their association to the RBC membrane. So far, it appears that the relative importance of the three enzymes is related to hydrogen peroxide levels within the RBC. In this chapter, we will focus on the importance of these peroxidases in the RBC's defense against OS mainly in the RBC cytosol and also the interplay between them and the RBC membrane. The potential role of their binding to the membrane will also be addressed.

Keywords: erythrocyte peroxidases, erythrocyte membrane, hemoglobin, oxidative stress, erythrocyte antioxidant system

1. Introduction

Erythrocytes are the most abundant cells in human blood, with unique morphology and metabolic characteristics and are highly important for body homeostasis. Erythrocytes come from a hematopoietic process—erythropoiesis—by which hematopoietic stem cells from the bone marrow proliferate and differentiate into mature red blood cells (RBCs) [1–3]. Erythrocytes are enucleated cells with a cytoplasm without organelles and rich in hemoglobin (Hb), which represents about 95% of total erythrocyte's cytoplasmic proteins [4, 5].

Membrane structure and composition are responsible for the biconcave disc shape and for the high deformability of the cell. These features are essential for oxygen transport, since RBCs have to undergo repeated shape changes without fragmentation, to assure their passage and oxygen perfusion through all vascular networks, namely, through capillary blood vessels with smaller lumen diameter than that of RBCs [6, 7]. Modifications in RBC membrane protein structure, by decreasing membrane flexibility and stability, may lead to premature removal of the cell reducing RBC's life span [1, 8].

The erythrocyte membrane is a complex structure composed by a lipid bilayer and a protein-based cytoskeleton tethered together by transmembrane proteins, such as protein band 3 and glycophorins. When under oxidative stress (OS) conditions, Hb is oxidized, it binds to the cytoplasmic domain of membrane protein band 3, triggering the formation of aggregates and the covalent linkage of natural anti-band 3 antibodies that may lead to premature RBC removal by splenic macrophages [1, 9].

Hb, the main cytoplasmic protein in RBC, is extremely important for erythrocyte's primary function, as a gas exchanger and for performing oxygen (O_2) distribution to body tissues. Erythrocytes carry O_2 from the lungs to the tissues and mediate carbon dioxide removal from the tissues to the lungs. In the lungs, O_2 binds to the heme group in Hb; in the tissues, O_2 is unloaded from Hb that undergoes a spatial rearrangement of the globin chains, allowing the entry of 2,3-diphosphoglycerate (2,3-DPG) which diminishes O_2 affinity [1, 2]. Oxyhemoglobin suffers autoxidation daily (2–3%), with oxidation of heme ferrous iron into ferric iron [10], leading to the formation of methemoglobin (metHb), which is not capable of O_2 transport, and the release of superoxide anion that is converted to H_2O_2, with a lower oxidant capacity [11]. The erythrocytes are capable of reducing metHb to functional Hb through methemoglobin reductases and of detoxifying the cell from H_2O_2 through the glutathione metabolism [2].

To prevent or reverse the harmful effects of OS, leading to oxidative changes in the erythrocyte constituents, RBCs are equipped with a powerful antioxidant system that is able to protect not only themselves, but also other cells and tissues while circulating throughout the vascular network. The protective antioxidant mechanisms of RBCs include enzymatic and non-enzymatic antioxidant systems that work together to detoxify the cell from reactive oxygen species (ROS) produced within or outside the cell.

In this chapter, we will focus on the importance of the RBC enzymatic antioxidant systems, namely on the peroxidases catalase (CAT), glutathione peroxidase (GPx) and peroxiredoxin 2 (Prx2). These peroxidases have a major role in the RBC's defense against OS, although the interplay between them is still a topic of discussion, as well as the potential role of their binding to the membrane, which may provide a protective mechanism for the cell.

2. Erythrocyte metabolism

Erythrocytes have a limited metabolic capacity since they lack a nucleus and organelles, like mitochondria, for oxidative metabolism [1, 11]. Therefore, energy is generated by the anaerobic glycolytic Embden-Meyerhof pathway, through which the breakdown of glucose to lactate generates two ATP molecules (**Figure 1**). This energy is essential for the maintenance of RBC's shape, membrane deformability and regulation of sodium-potassium pump [1, 2]. This pathway also provides NADH, which is important as a cofactor of methemoglobin reductase to regenerate oxidized Hb to its reduced functional state. The Luebering-Rapoport shunt, a side arm of Embden-Meyerhof pathway, produces 2,3-DPG (**Figure 1**), essential for the regulation of O_2 affinity [1, 2]. Around 80–90% of glucose that enters the cell follows the Embden-Meyerhof pathway, while about 10% is metabolized through the pentose phosphate pathway [12] to ribose-5-phosphate concomitantly generating NADPH (**Figure 1**). NADPH is essential for glutathione (GSH) metabolism that assures the detoxification of RBCs from ROS, being, therefore, an important erythrocyte antioxidant defense mechanism [11].

GSH is a tripeptide constituted by the three amino acids L-glutamate, L-cysteine and L-glycine [13, 14], existing in the cell in two different forms, the reduced form

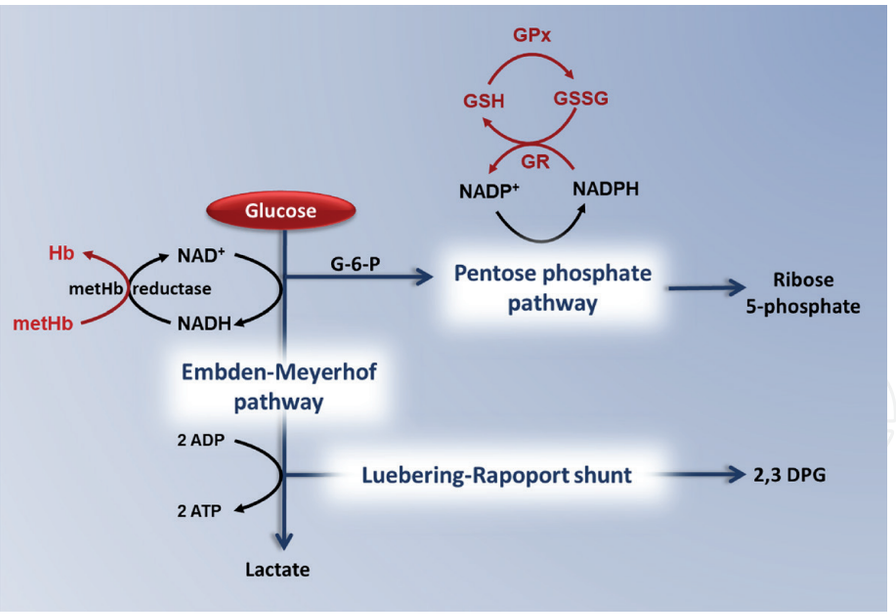

Figure 1.
Erythrocyte metabolic pathways synopsis. 2,3 DPG, 2,3-diphosphoglycerate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; G-6-P, glucose 6-phosphate; GPx, glutathione peroxidase; GR, glutathione reductase; GSSG, oxidized glutathione; GSH, glutathione; Hb, hemoglobin; metHb, methemoglobin; NAD, NADH, nicotinamide adenine dinucleotide; NADP, NADPH, nicotinamide adenine dinucleotide phosphate.

(GSH) and the oxidized form (GSSG). The reduced form is the predominant one and GSSG is maintained at low levels, less than 1% mainly by the action of NADPH-dependent glutathione reductase [13], which converts GSSG into the reduced GSH (**Figure 1**). Despite the limited biosynthesis capability of the RBC, some endogenous GSH is still synthetized in the cytosol through two ATP-dependent reactions catalyzed by two different enzymes, glutamate cysteine ligase and glutathione synthase [13].

As an antioxidant defense, GSH has several roles: it can directly scavenge hydroxyl radicals and peroxynitrites [14, 15]; it is involved in lipid peroxide detoxification [16]; it can reduce H_2O_2 in the presence of GPx by the reduction of its thiol group and keeps thiol groups from Hb, enzymes and membrane proteins in the reduced form [13], which is very important for the preservation of their functions, once oxidation of these groups can lead to functional and structural cellular modifications. Therefore, the GSH/GSSG ratio is an important indicator of the cell redox state [15].

In OS conditions, the capacity of RBCs to reduce GSSG to GSH decreases, leading to GSSG accumulation and, consequently, to GSH depletion [14]. Diminished GSH concentrations have been described in physiological events, as aging, and in pathologic conditions associated with OS, such as Alzheimer's disease, Parkinson's disease [15], sickle cell anemia and asthma [17, 18].

Ascorbic acid (vitamin C) and α-tocopherol (vitamin E) obtained mostly from diet are also important non-enzymatic erythrocyte antioxidants [19]. α-Tocopherol has a protective effect on RBC membranes against lipid peroxidation [11, 19]. Ascorbic acid can reduce O_2^- levels and it is an important regenerator of α-tocopherol. Uric acid can also act as an antioxidant and is able to directly scavenge OH^- [12].

3. Oxidative stress in erythrocytes

During their life span, the erythrocytes are continuously exposed to high O_2 tension, due to their primary function as gas carriers, and are unable to synthesize new or repair damaged proteins, due to the lack of nucleus and other organelles. Therefore, RBCs are more vulnerable to ROS action than other cells of the human body [12].

ROS are chemically reactive species containing oxygen with one or more unpaired electrons that are formed by the reduction of an O_2 molecule (**Figure 2**) [12, 20, 21]. The transfer of one electron to an O_2 molecule produces superoxide anion (O_2^-), the precursor of other ROS [22]. Spontaneous O_2^- dismutation or catalysis by superoxide dismutase (SOD) action produces hydrogen peroxide (H_2O_2) [22]. This molecule is not a free radical and is more stable than O_2^-, but it can easily cross cell membranes and cause damage in other cells and tissues [12]. The RBC needs to be detoxified from H_2O_2, as its accumulation leads to the production of other more potent ROS. This molecule can be decomposed into water and O_2 by CAT, GPx or Prx2. In case of failure of these antioxidant enzymes, H_2O_2 can also be reduced to hydroxyl radical (OH^-), the most harmful free radical for biological systems, due to its high reactivity. With a short half-life, OH^- does not travel far, but has a much higher oxidant potential than all the other ROS [12, 20].

OS arises when an imbalance between free-radical formation and antioxidant defenses occurs, that is, when ROS concentration overwhelms the antioxidant capacity within the RBC [19]. The endogenous source of ROS in erythrocytes is the autoxidation of Hb [11, 12]; occasionally oxyhemoglobin loses one electron (2–3% per day) leading to the production of O_2^- and oxidized Hb (metHb) (**Figure 2**) which is not able to bind and carry O_2. Erythrocytes can also develop OS due to exogenous ROS that are able to diffuse and cross the RBC membrane. The enhanced production and release of ROS by activated inflammatory cells, macrophages, neutrophils and endothelial cells [23], are the main source of exogenous ROS. The continuous exposure of RBCs to ROS can cause cell damage, including lipid and protein oxidation, causing damages in enzymes and ion transport proteins [19, 24].

Considering the major role of Hb, its oxidation may trigger important structural and functional changes in RBCs [11, 12]. Thus, as oxidation of Hb occurs, even under normal physiological conditions, the antioxidant defenses have a crucial role in the regeneration of functional Hb and maintenance of low metHb

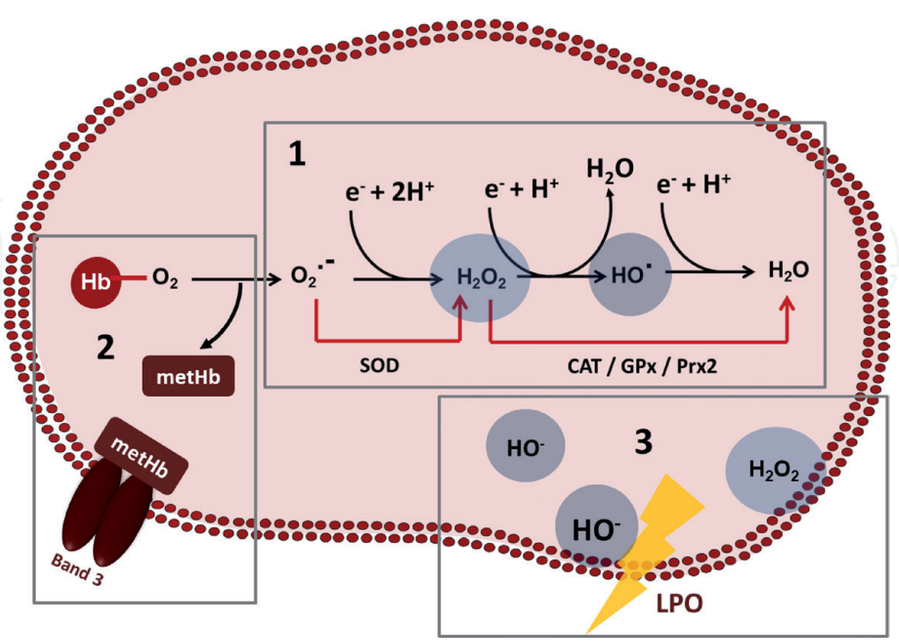

Figure 2.
Oxidative stress in erythrocytes. (1) Production of reactive oxygen species resulting from hemoglobin autoxidation. (2) Linkage of denatured Hb to erythrocyte membrane band 3 protein. (3) Peroxidation of erythrocyte membrane lipids. CAT, catalase; e^-, electron; GPx, glutathione peroxidase; H^+, hydrogen; H_2O, water; H_2O_2, hydrogen peroxide; Hb, hemoglobin; HO^-, hydroxyl radical; LPO, lipid peroxidation; metHb, methemoglobin; O_2, oxygen; O_2^-, superoxide anion; Prx2, peroxiredoxin 2; SOD, superoxide dismutase.

levels [11]. When oxidized, the primary structure of Hb is altered by the establishment of disulfide cross-links between globin chains that make the molecule unstable, leading to the formation of Heinz bodies and, eventually, to a premature RBC removal [11]. Indeed, oxidized Hb binds to the cytoplasmic domain of band 3 protein in the RBC membrane (**Figure 2**), triggering band 3 clustering, marking the erythrocyte for removal by splenic macrophages [23, 25]. Clustering of band 3 as a result of enhanced metHb formation and linkage to the membrane has been reported in several erythrocyte disorders such as, hereditary spherocytosis [26], beta-thalassemia [27], sickle cell anemia [28] and glucose-6-phosphate dehydrogenase deficiency [29]. An increase in metHb levels and in its linkage to the RBC membrane, accompanied by ROS formation, was also found in stored RBCs for blood transfusion [30] and in exogenous H_2O_2-induced OS upon healthy erythrocytes [31, 32]. Hb oxidation also occurs as a natural process, resulting from RBC aging [33], that is associated with metabolic degradation due to reduction in enzyme activity.

RBC membrane is an important target for both endogenous and exogenous ROS that may induce oxidative changes in membrane proteins and lipids. Changes in RBC membrane proteins have been reported in some diseases in which OS is involved, such as chronic kidney disease [34, 35] or chronic obstructive pulmonary disease [36]. ROS can affect erythrocyte proteins through oxidation of the protein backbone, cross-linking or amino acid oxidation [19, 24]. The polyunsaturated fatty acids (PUFAs) of the RBC cell membranes are highly vulnerable to oxidation (about half of the RBC membrane fatty acids are unsaturated [12]). ROS are able to break the double bonds of PUFA, producing malondialdehyde (MDA) [24], the main end-product of membrane lipid peroxidation (LPO). MDA is a highly reactive molecule that can further react with lipids and proteins of the membrane. These changes in membrane proteins and lipids contribute to functional and structural alterations that decrease erythrocyte membrane stability and deformability and trigger premature RBC removal [12, 24]. LPO has also been described following metHb binding to the membrane, suggesting that this linkage favors LPO [37]. Increased LPO and MDA levels have been reported in different conditions associated to OS, including physiological events, such as aging [38], and pathological conditions like schizophrenia [39], Alzheimer's disease [40], inflammatory associated diseases [41], atherosclerosis [42] and chronic kidney disease [43]. Considering the reduced biosynthetic capacity of erythrocytes, they accumulate oxidative changes along their life span and, therefore, the OS-induced changes in RBCs could be used as useful biomarkers in several pathological and physiological conditions.

4. Erythrocyte peroxidases

To cope with oxidative injuries, the erythrocytes have several enzymes that neutralize ROS or transform them into less reactive species. SOD provides the first line of protection against free radicals. It is a cytosolic copper-zinc containing enzyme that converts O_2^- into the less reactive H_2O_2 (Eq. (1)), through the alternate reduction and re-oxidation of Cu^{2+} [44].

$$2\,O_2^{\cdot-} + 2\,H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$

Afterward, H_2O_2 can be decomposed into O_2 and water by three distinct erythrocyte peroxidases: CAT, GPx and Prx2 [45–47].

4.1 Catalase

Catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) is an intracellular enzyme found at high concentrations in erythrocytes and liver peroxisomes in mammals [48–51]. CAT is a very important enzyme, as it is able to protect cells and tissues from the toxic effects of H_2O_2 [19, 51]. As referred, the decomposition of H_2O_2 is particularly important in erythrocytes, to prevent oxidation of Hb and of other RBC constituents. CAT is one of the most efficient enzymes, since it exhibits one of the fastest turnover rates with a capacity to convert millions of H_2O_2 molecules per second (kcat = 4×10^7 s^{-1}) [45, 48].

More than 300 catalase sequences are available, divided among several groups [45, 50, 52]. Human erythrocyte catalase, a tetrameric protein of 244 kDa [53], belongs to the monofunctional heme-containing catalases. Each monomer is formed by a single polypeptide chain that has a molecular weight of approximately 60 kDa [54]. Each subunit also has one heme group at the catalytic center, with iron (III) linked to protoporphyrin IX [53]. Some studies [55–57] showed that each catalase tetramer has four tightly bound NADPH molecules that appear to be important only to protect the enzyme against inactivation by its own substrate (H_2O_2), and are not essential for its catalytic activity. It is thought that NADPH prevents the formation of the inactive form of catalase (Compound II) and that it increases the rate of removal of this inactive form [45, 53, 55, 56].

The overall reaction catalyzed by CAT involves the degradation of two molecules of H_2O_2 to two molecules of water and one of O_2 (Eq. (2)).

$$2\,H_2O_2 \rightarrow 2\,H_2O + O_2 \qquad (2)$$

The H_2O_2 decomposition is believed to occur in two steps (**Figure 3**, steps 1 and 2) [45, 50, 52]. The first involves the interaction between one molecule of H_2O_2 and CAT which leads to the production of Compound I, in which the heme group is oxidized to oxyferryl species [45, 50, 52]. Compound I is an enzymatic active form

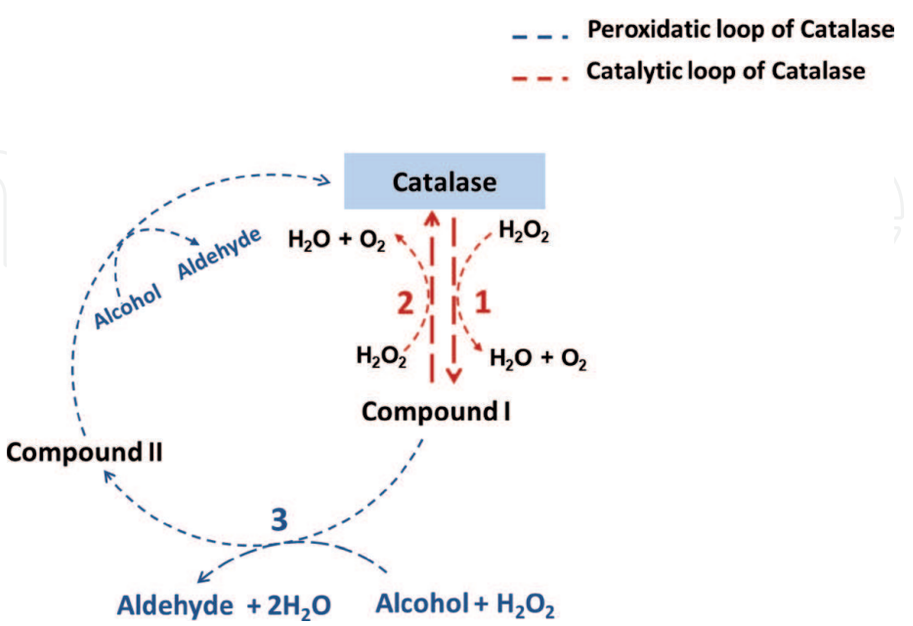

Figure 3.
Hydrogen peroxide removal by catalase. (1) Interaction between H_2O_2 and catalase leading to the production of Compound I. (2) Interaction of a second H_2O_2 molecule with Compound I producing one molecule of H_2O, O_2 and the enzyme at the resting state. (3) Catalase peroxidatic activity. H_2O, water; H_2O_2, hydrogen peroxide; O_2, oxygen.

of catalase but spectroscopically different [58]. At the second step, a second H_2O_2 molecule acts, as a reducing agent, on Compound I, producing one molecule of water, one of O_2 and the enzyme in the resting state [45, 50, 52].

In addition to their catalytic activity, catalases can also function peroxidatively (**Figure 3**, step 3) to eliminate H_2O_2 [45, 49]. In this case, the enzyme uses peroxidation to eliminate H_2O_2 molecules by oxidizing substances like alcohols. The peroxidatic activity of CAT is, usually, minor, weak and restricted to smaller substrates, as compared to other peroxidases [45].

When compared with the other H_2O_2 scavenger enzymes, CAT seems to be the key enzyme to remove high intracellular concentrations of H_2O_2 [32, 53, 59, 60]. Moreover, CAT is highly specific for its substrate, H_2O_2, and it is not able to eliminate organic peroxides, unlike other peroxidases [59].

Catalase has also been studied in a number of different diseases in which OS is implicated, such as, diabetes mellitus where patients presented lower CAT values [61]; in some type of cancers, CAT activity was lower in patients, especially in lymphomas, when compared with CAT activity in the normal population [62] and, in bipolar disorder, subjects with bipolar depression presented a significant increase in CAT levels [63].

4.2 Glutathione peroxidase

GPx (GSH_2:H_2O_2 oxidoreductase, EC 1.11.1.9) is an intracellular antioxidant enzyme that contributes to prevent H_2O_2 accumulation in cells. In mammals, eight GPxs have been identified [47] at different locations and cellular compartments, differing at their catalytic center. GPx-1 is one of the most abundant type of GPx and the only type present in RBC's cytosol [60]. GPx-1 is a tetramer of four identical subunits of 21 kDa [64], each with one selenocysteine (Sec) [65]. The catalytic tetrad formed by Sec, glutamine, tryptophan and asparagine is essential for GPx activity, since these residues are crucial for enzyme-substrate interaction and stabilization of the GSH-GPx interaction [47, 65, 66].

GPx-1 catalyzes the reduction of H_2O_2 [47, 66], lipid hydroperoxides and other low molecular hydroperoxides [64] into water, or into the corresponding alcohols, using GSH as a reducing agent; thus, GPx-1 prevents both lipid peroxidation [65, 67] and H_2O_2 accumulation.

The overall catalytic reaction of GPx-1 is given by Eq. (3).

$$ROOH + 2GSH \rightarrow ROH + H_2O + GSSG \quad (3)$$

The catalytic cycle of GPx includes a peroxidatic part that is followed by a reductive step (**Figure 4**) [47]. In the peroxidatic part, one molecule of H_2O_2 reacts with the selenol group from Sec in GPx, producing a selenenic acid at the active site [47, 66]. In the reductive part, one GSH molecule forms a selenadisulfide bond with the selenic acid forming the glutathiolated selenol intermediate [47]. As a second GSH molecule reduces the glutathiolated selenol bond, GSSG is released and GPx is regenerated. The restoration of GSH involves the action of the NADPH-dependent enzyme, glutathione reductase. The recycling of NADPH associates the GSH system to the pentose-phosphate pathway [66].

CAT was considered as the only enzyme involved in erythrocyte antioxidant defense by performing H_2O_2 removal [68]. Nowadays, it is known that GPx also has a major role in RBC antioxidant protection, being essential for the detoxification of low H_2O_2 concentrations and hydroperoxides [59, 69, 70], with a constant rate superior to 10^7 M^{-1} s^{-1} [47, 71].

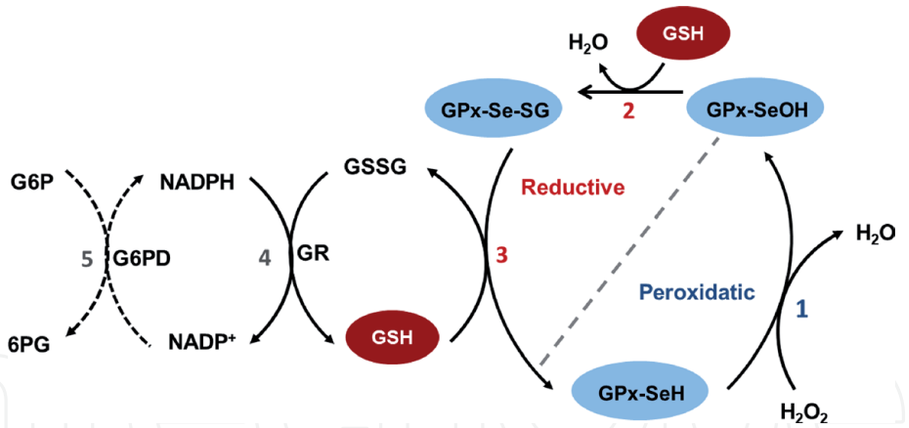

Figure 4.
Catalytic cycle of glutathione peroxidase 1. (1) Peroxidatic part of GPx-1 catalytic cycle. (2) and (3) Reductive part of GPx-1 catalytic cycle (4) Regeneration of GSH by NADPH-dependent GR. (5) $NADP^+$/NADPH recycling by G6PD. 6PG, 6-phosphogluconolactone; G6P, glucose-6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; GPx-SeH, glutathione peroxidase selenol; GPx-SeOH, glutathione peroxidase selenic acid; GPx-Se-SG, glutathiolated selenol intermediate; GR, glutathione reductase; GSH, glutathione; GSSG, oxidized glutathione; H_2O, water; H_2O_2, hydrogen peroxide; NADPH/$NADP^+$, nicotinamide adenine dinucleotide phosphate.

4.3 Peroxiredoxin 2

Peroxiredoxins (Prxs; SH:H_2O_2 oxidoreductases, EC 1.11.1.15) are a family of homodimeric peroxidases with an antioxidant role in living organisms. Six different mammalian Prx isoforms are known (Prx 1–6). Prx 1 and Prx 6 can be found in erythrocytes, although in much lower amounts than Prx2, which is the third most abundant protein in the RBC cytosol (after Hb and carbonic anhydrase) [5].

For a long time, CAT and GPx were considered the major erythrocyte players for H_2O_2 detoxification [68]. However, several studies [72–75] have shown the significant role of Prx2 as an efficient H_2O_2 scavenger in the erythrocyte antioxidant system. Studies using Prx2 knock-out mice showed that these animal models developed hemolytic anemia and their erythrocytes displayed a significantly shorter life span, when compared to wild-type mice [72]. In contrast, CAT and GPx knock-out mice showed a normal hematologic profile and normal development [72, 76]. Another important study reported that Prx2 reacts with H_2O_2 at a constant rate ($1.3 \times 10^7\ M^{-1}\ s^{-1}$) comparable with that of CAT and GPx [75].

Under its physiological functional state, Prx2 appears as a monomer (active form) of about 20–30 kDa and when interacting with H_2O_2, Prx2 is oxidized and a disulfide-linked dimmer is formed (inactive form) [73, 77]. This oxidized form is reversed by thioredoxin (Trx)/Trx reductase/NADPH system, although, in RBCs, it is a very slow regeneration due to the low concentrations of Trx reductase [73]. Besides H_2O_2, Prx2 can also remove peroxynitrites [5] and hydroperoxides in the RBC membrane [73, 75].

Since Prx2 is a thiol-dependent peroxidase, it uses redox-active cysteines to reduce peroxides. According to the number and location of the catalytic cysteines, Prxs are divided into three classes: the typical 2-Cys, the atypical 2-Cys and the 1-Cys [46]. Prx2 is a typical 2-Cys peroxiredoxin, with two redox-active cysteines: the peroxidatic cysteine near residue 50 in one subunit and the resolving cysteine near residue 170 in the other subunit [46]. The overall peroxidase reaction is given by Eq. (4).

$$\mathbf{ROOH + 2e^- \rightarrow ROH + H_2O} \qquad (4)$$

The catalytic cycle of Prx2 is composed by two steps (**Figure 5**). The first step is the oxidation of peroxidatic cysteine to peroxidatic cysteine-sulfenic acid by interaction with H_2O_2. In the second step, the resolving cysteine of one Prx subunit attacks the peroxidatic cysteine-sulfenic acid of the other subunit generating an inter-subunit disulfide bond [46, 75]. This dimeric form of Prx2 is non-functional, but the disulfide bridge between the subunits can be broken by Trx, regenerating Prx2, and completing the catalytic cycle [73]. In turn, Trx can be reduced by the NADPH-dependent Trx reductase. Reduction of the disulfide bond by Trx is the rate-limiting step in the Prx2 catalytic cycle [73].

In the presence of high peroxide levels, 2-Cys Prxs can become over-oxidized to their sulfinic acid form. In RBCs, this hyperoxidation of Prx2 does not occur, as it is counteracted by sulfiredoxin [60, 73].

As part of the erythrocyte antioxidant system, Prx2 is responsible for the removal of low H_2O_2 concentrations, since the Trx system has a limited capacity for Prx2 regeneration into its reduced active form [32, 59, 60, 73]. Recently, it was found that Prx2 can have a dual function according to H_2O_2 levels, as an antioxidant enzyme or as a chaperone, due to changes in its structure [59, 73, 78]. In RBCs, Prx2 can bind to Hb under OS conditions to stabilize its structure and prevent Hb aggregation [79]. A recent work by our group [80] showed that under steady-state conditions, Prx2 acts as a typical peroxidase, protecting the erythrocytes from low endogenous levels of ROS. However, when RBCs are saturated with carbon monoxide, Prx2 was observed only in the active form in the cytosol and none in the oxidized form, suggesting that Prx2 is acting specifically to protect Hb, shifting its function from peroxidase to chaperone. Prx2, initially called calpromotin, is also required to regulate the calcium-dependent potassium channel in the erythrocyte membrane [81].

The use of Prx2 as a potential therapeutic drug target has gained growing interest; so far, it has already been reported as a possible target for malaria treatment [82]. Changes in human Prx2 expression or oxidation state have been associated with several diseases: alterations in Prx2 expression have been reported in different types of cancer [83, 84]; oxidatively modified Prx2 has been found in Alzheimer's disease patients [85]; hyperoxidized forms of Prx2 were also found in asthmatic patients [86] and linkage of cytosolic Prx2 to the RBC membrane was found in

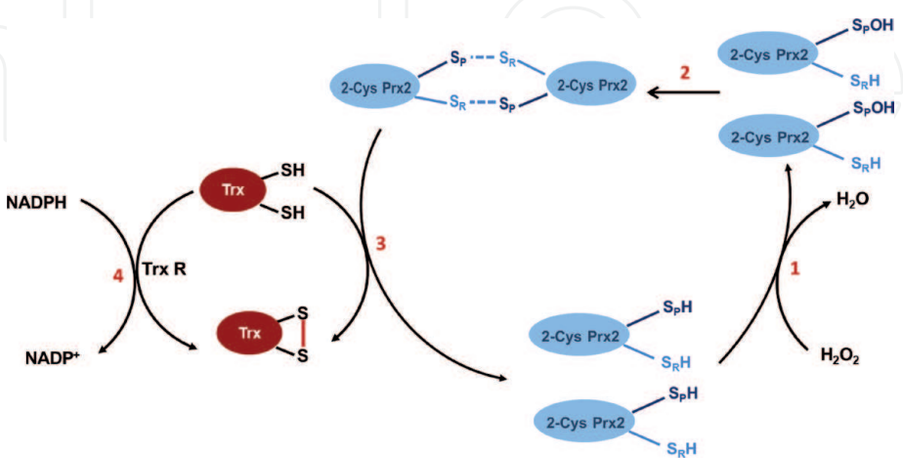

Figure 5.
Peroxiredoxin 2 catalytic cycle. (1) Oxidation of S_PH to S_POH by interaction with H_2O_2. (2) Attack of S_RH of one subunit to S_POH of the other subunit and formation of the intersubunit disulfide bond. (3) Reduction of the disulfide bond by Trx. (4) Regeneration of reduced Trx by NADPH-dependent Trx reductase. 2-Cys Prx, 2-cys peroxiredoxin 2; H_2O, water; H_2O_2, hydrogen peroxide; NADPH/NADP$^+$, nicotinamide adenine dinucleotide phosphate; S_PH, peroxidatic cysteine; S_POH, peroxidatic cysteine sulfenic acid; S_RH, resolving cysteine; Trx, thioredoxin; TrxR, thioredoxin reductase.

hereditary spherocytosis patients [26]. Thus, there has been an increasing interest in Prx2 as a biomarker for different conditions where OS plays a crucial role. For example, a novel HPLC method to monitor the levels of reduced Prx2 form was developed [87], which could prove useful for future clinical practice.

5. Interplay between erythrocyte peroxidases and the erythrocyte membrane

The individual contribution of CAT, GPx and Prx2 to erythrocyte protection against H_2O_2 damage has been a controversial issue for many years. It is clear that all three enzymes are involved in the prevention of H_2O_2 accumulation in the RBC through H_2O_2 conversion into water and O_2 (**Figure 6**); however, the relative importance of the three enzymes is still a topic of discussion.

CAT was considered the main erythrocyte defense against OS, for many years [68, 88], but several studies [59, 69, 89] showed that GPx has also an important role in H_2O_2 decomposition. In fact, a study by Johnson et al. [59] showed that CAT and GPx-deficient RBCs were more sensitive to H_2O_2-induced OS than cells with only CAT deficiency, suggesting that GPx has an important role in erythrocyte defense. The same authors also showed [59, 70] that the action of both CAT and GPx was insufficient to explain the erythrocyte oxidative catabolism, and proposed [70] a model including Prx2 that, in accordance with their experimental data, could better explain the erythrocyte antioxidant defense system. Furthermore, the development of hemolytic anemia in Prx2 knock-out mice [72–74] and the high turnover rate of Prx2 with H_2O_2 [5, 75] strengthened the importance of the role of Prx2 in RBC antioxidant protection.

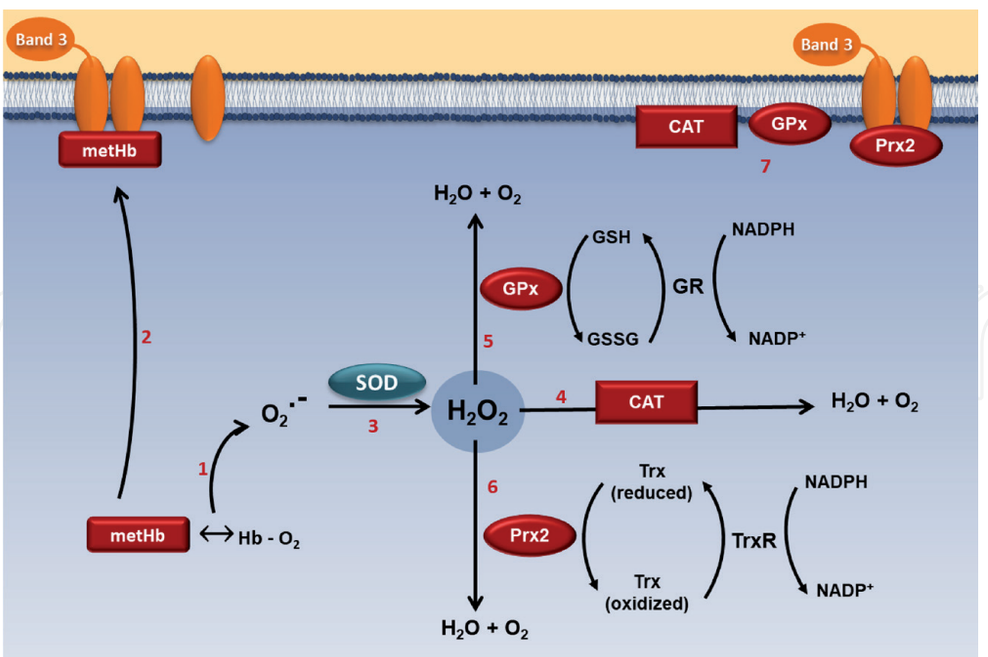

Figure 6.
Interplay between erythrocyte's peroxidases. (1) Methemoglobin formation and release of O_2^-. (2) Formation of band 3 protein aggregates triggered by methemoglobin linkage to the integral membrane protein band 3. (3) O_2^- removal by SOD with H_2O_2 formation. (4) H_2O_2 removal by CAT. (5) H_2O_2 removal by GPx. (6) H_2O_2 removal by Prx2. (7) Linkage of CAT, GPx and Prx2 to the RBC membrane imposed by oxidative stress. CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, oxidized glutathione; H_2O, water; H_2O_2, hydrogen peroxide; Hb, hemoglobin; metHb, methemoglobin; Hb-O_2, oxyhemoglobin; NADPH/NADP+, nicotinamide adenine dinucleotide phosphate; O2, oxygen; O_2^-, superoxide anion; Prx2, peroxiredoxin 2; SOD, superoxide dismutase; Trx, thioredoxin; TrxR, thioredoxin reductase.

The relative importance of the three enzymes appears to be related to the H_2O_2 levels in the RBC [59]. CAT is able to scavenge exogenous and high endogenous peroxide levels [59, 60, 70], while GPx and Prx2 appear to scavenge endogenous and low peroxide levels [59, 70, 73]. Thus, the elimination of the basal flux and low H_2O_2 levels is performed by GPx and Prx2, since CAT does not work efficiently at low H_2O_2 levels [60]. Whenever RBCs are exposed to higher H_2O_2 levels, CAT becomes essential for its rapid removal since this enzyme has a high turnover rate, unlike GPx and Prx2 that become less efficient (or even inactive), due to their slow GSH reductase and Trx recycling systems, respectively [59, 60, 73].

The enzymes GPx and Prx2 seem to have other functions in RBC antioxidant defense, beyond H_2O_2 scavenging. In fact, GPx and Prx2 are also able to detoxify organic peroxides [5, 59, 75], while CAT does not show this function [59]. As shown by Johnson et al. [59, 90], GPx-deficient RBCs are more susceptible to oxidation by organic peroxides than wild type cells [90]; and when CAT deficiency was added to GPx deficiency, no increased sensitivity to oxidation by organic peroxides occurred in these cells [59]. Thus, when erythrocytes are exposed to high H_2O_2 levels, organic peroxides will accumulate, since GPx and Prx2 become less efficient and CAT is not able to detoxify these organic peroxides [60].

Recently, it was reported that Prx2 can have multiple functions, as a peroxidase or as a chaperone, through the formation of high-molecular-weight complexes [78, 91]. It was shown that Prx2 acts as a chaperone in RBCs, by interacting directly with Hb to maintain its stability [79]. A study by our group [80] showed that when erythrocytes were saturated with CO, the enzyme Prx2 was present in the cytosol only in the monomeric form, suggesting that Prx2 was not acting as a peroxidase but, instead, exclusively as a chaperone for Hb's protection. Several authors have suggested [59, 92] that Prx2 can also have an important role in erythropoiesis. Johnson et al. [59] believe that the role of Prx2 as Hb chaperone is especially important in the different stages of erythropoiesis. According to Matte et al., Prx2 appears to be a regulator of iron homeostasis during erythropoiesis [92].

CAT, GPx and Prx2 are essentially cytosolic enzymes; however, the association of these enzymes to the erythrocyte membrane has been reported in different in vivo and in vitro studies [26, 31, 32, 93–97]. Erythrocytes from patients with hereditary spherocytosis showed CAT [95] and Prx2 bound to their membranes [26]. The association of CAT to the membrane appears to be a consequence of the metabolic stress triggered by the destabilization of membrane structure, due to an altered RBC membrane composition; the linkage of Prx2 might be involved in the protection of the RBC membrane against LPO [26, 31]. This linkage of Prx2 to the membrane appears to be through the N-terminal cytoplasmic domain of band 3, which is also the site of linkage of other cytoplasmic proteins, including metHb [98]. In recent in vitro assays performed by our group [32], we showed that H_2O_2-induced oxidative stress triggered the binding of Prx2 and GPx to RBC membrane. A recent study by Bayer et al. [96] about the interaction of Prx2 with the RBC membrane reported that the linkage of Prx2 to the membrane is independent of its redox state and that Prx2 competes with Hb for the same binding site in the RBC membrane. Thus, they demonstrated that Prx2 prevents metHb aggregation, and, probably acts as a chaperone for the denatured Hb [96]. Contrary to what was previously observed [26, 31, 32], Bayer et al. [96] found a decrease in Prx2 membrane binding, with increasing concentrations of H_2O_2. A decrease in Prx2 linkage to the RBC membrane with OS conditions was also observed in beta-thalassemic mice RBCs, probably due to the increase in metHb that binds to the membrane, reducing the access of Prx2 to the same site [99].

The linkage of GPx to the RBC membrane was first described by van Gestel et al., using proteome analysis [94]. Later on, Rocha et al. showed the linkage of GPx to the RBC membrane in response to in vitro H_2O_2-induced OS [32].

Studies of stored RBCs in blood bank conditions also reported the recruitment of Prx2, CAT and GPx to the RBC membrane due to OS modifications resulting from the metabolic stress of long-term erythrocyte storage [93, 97].

Data in literature suggest that the linkage of these RBC cytosolic enzymes to the membrane is triggered by metabolic stress, possibly, to protect the erythrocyte membrane and counteract the effects of OS.

6. Conclusions

Erythrocytes, as oxygen carrier cells, are highly exposed to oxidative injury; to face this challenge, RBCs are well equipped with an efficient antioxidant system, important to maintain erythrocyte homeostasis during its life span. The antioxidant system includes non-enzymatic and enzymatic agents such as peroxidases, namely Prx2, GPx and CAT. The role and interplay between these enzymes that prevent H_2O_2 accumulation in the erythrocyte has been a topic of discussion over the years. So far, it appears that their role depends on the H_2O_2 levels within RBC: CAT is crucial for scavenging high exogenous and endogenous peroxide levels, GPx and Prx2 are important for scavenging low endogenous and low peroxide levels. GPx and Prx2 are also able to detoxify the cell from organic peroxides, unlike CAT that does not show this function. Therefore, GPx and Prx2 can have a direct role on RBC membrane antioxidant defense.

Several authors have already reported the linkage of CAT, GPx and Prx2 to the erythrocyte membrane in case of metabolic stress and/or OS. In fact, the recruitment of the three peroxidases to the RBC membrane has been described in OS-associated conditions, by in vitro assays and by studies with stored RBCs under blood bank conditions.

Studies about Prx2 reveal a dual function in RBC defense, as a peroxidase and as an Hb chaperone preventing metHb aggregation. Some authors have also proposed that Prx2 may have a major role in erythropoiesis.

Erythrocytes are the ultimate antioxidant defense against the harmful effects of OS in humans; so, the knowledge about the RBC antioxidant system has evolved over time, and should continue to grow, focusing on the importance of CAT, GPx and Prx2 working together in ROS detoxification and also their potential role in the erythrocyte membrane.

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Conflict of interest

The authors report no conflict of interest.

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Author details

Daniela Melo[1], Susana Rocha[1], Susana Coimbra[2,3] and Alice Santos Silva[1*]

1 UCIBIO, REQUIMTE, Laboratory of Biochemistry, Department of Biological Sciences, Faculty of Pharmacy, University of Porto, Porto, Portugal

2 UCIBIO, REQUIMTE, Porto, Portugal

3 CESPU, Institute of research and Advanced Training in Health Sciences and Technologies (IINFACTS), Gandra-Paredes, Portugal

*Address all correspondence to: assilva@ff.up.pt

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