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Original Contribution

# Peroxiredoxin-2 expression is increased in β-thalassemic mouse red cells but is displaced from the membrane as a marker of oxidative stress

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ABSTRACT

Peroxiredoxin 2 (Prx2), the third most abundant cytoplasmic protein in red blood cells (RBCs), is involved in the defense against oxidative stress. Although much is known about Prx2 in healthy RBCs, its role in pathological RBCs remains largely unexplored. Here, we show that the expression and net content of Prx2 are markedly increased in RBCs from two mouse models of β-thalassemia (β-thal; $Hbb^{th/th}$ and $Hbb^{th3/+}$ strains). We also demonstrate that the increased expression of Prx2 correlates with the severity of the disease and that the amount of Prx2 bound to the membrane is markedly reduced in β-thal mouse RBCs. To explore the impact of oxidative stress on Prx2 membrane association, we examined Prx2 dimerization and membrane translocation in murine RBCs exposed to various oxidants (phenylhydrazine, PHZ; diamide; $H_2O_2$). PHZ-treated RBCs, which mimic the membrane damage in β-thal RBCs, exhibited a kinetic correlation among Prx2 membrane displacement, intracellular methemoglobin levels, and hemichrome membrane association, suggesting the possible masking of Prx2 docking sites by membrane-bound hemichromes, providing a possible mechanism for the accumulation of oxidized/dimerized Prx2 in the cytoplasm and the increased membrane damage in β-thal RBCs. Thus, reduced access of Prx2 to the membrane in β-thal RBCs represents a new factor that could contribute to the oxidative damage characterizing the pathology.
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Peroxiredoxins are ubiquitous proteins involved at least in part in the defense against oxidative stress through their ability to reduce and detoxify a vast range of organic peroxides, $H_2O_2$, and peroxynitrite [1–3]. In red cells, peroxiredoxin-2 (Prx2)[1] is the third most abundant cytoplasmic protein and a typical 2-cysteine peroxiredoxin. Erythrocyte Prx2 (formerly termed calpromotin) was initially characterized as a membrane-associated protein whose reversible binding to the membrane was linked to regulation of the $Ca^{2+}$-activated $K^+$ channel (Gardos channel) via a still unknown mechanism [1,4–11]. In a mouse model of sickle cell disease, interaction of Prx2 with the membrane was further shown to be modulated during hypoxia-induced acute sickle cell-related vaso-occlusive crisis [12]; however, a function of this reversible association was not established. Recently Rocha et al. reported specific binding of Prx2 in patients with hereditary spherocytosis [9]. In Prx2 knockout mice, elimination of erythrocyte Prx2 has, moreover, been demonstrated to induce a hemolytic anemia associated with signs of red cell oxidative damage [13].

β-Thalassemia is a common inherited red cell disorder caused by the reduced or absent synthesis of the β chain of hemoglobin. The resulting imbalance between α and β chains leads to precipitation of α-globin chains on the membrane, which induces membrane damage and a shortening of erythrocyte life span [14–18]. Red cells from β-thalassemia intermedia patients show membrane clusters of hemichromes and band 3, presumably as a consequence of oxidative injury [14–20]. Immunoglobulins and complement components localize on the membrane surface over these clusters, mediating the removal of the damaged erythrocytes by macrophages. Membrane lipid peroxidation, loss of phospholipid asymmetry, and externalization of phosphatidylserine have been also documented in β-thalassemic erythrocytes [16,18,21]. Importantly, mouse models of β-thalassemia show most of the pathologic features of their human counterparts.

In our studies of Prx2 behavior in β-thalassemia, we have focused on two mouse models of the disease. In the first model, spontaneous deletion of the *b1* gene ($Hbb^{th/th}$) results in the absence of β-major

*Abbreviations:* Prx2, peroxiredoxin 2; β-thal, β-thalassemia; RBC, red blood cell; PHZ, phenylhydrazine; MetHb, methemoglobin; Hb, hemoglobin.

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globin synthesis that is accompanied by a partial compensatory increase in β-minor globin chain synthesis. The derived erythrocytes are characterized by a net ~25% reduction in β chain expression, resulting in a mild β-thalassemia. In the second model, heterozygous deletion of both *b1* and *b2* loci ($Hbb^{th3/+}$), without any compensatory production of adult β-globin chains, is seen to result in an ~50% decrease in β-globin synthesis [20,22], leading to a more severe form of the disease. Previous studies of these two β-thalassemic mouse models have suggested that $Hbb^{th/th}$ most closely resembles the mild phenotype of human β-thalassemia intermedia, whereas the anemia, splenomegaly, and bone abnormalities of $Hbb^{th3/+}$ are more similar to the pathologic symptoms of severe human β-thalassemic heterozygotes. In both models as well as in human cases of β-thalassemia, oxidative damage to the membrane is believed to cause many of the pathologic features of the disease.

Here, we show that Prx2 expression and content were higher in red cells from β-thalassemic mouse models than in wild-type mice but the amount of Prx2 associated with the membrane was markedly reduced. We showed that Prx2 responds differently to various oxidants (phenylhydrazine, PHZ; diamide; and $H_2O_2$) and partitions abnormally between the membrane and the cytosol in response to different oxidative stresses. The translocation to the membrane of either native Prx2 or Prx2 from oxidant-treated red cells was markedly reduced when the red cell membranes were treated with PHZ to generate β-thalassemic-like red cell membrane damage. The intercorrelations among Prx2 membrane binding, methemoglobin (MetHb) formation, and hemichrome membrane binding together with the accumulation of oxidized/dimerized Prx2 in the cytoplasm might affect Prx2 antioxidant function similarly on β-thal and PHZ-treated membrane proteins and represent a new factor contributing to the oxidative damage characterizing β-thalassemia.

## Material and methods

### *Drugs and chemicals*

NaCl, KCl, $Na_2HPO_4$, $Na_3VO_4$, $KH_2PO_4$, $MgCl_2$, $NH_4HCO_3$, Mops, Tris, *N*-ethylmaleimide (NEM), choline chloride, benzamidine, β-mercaptoethanol, glycine, bromphenol blue, trypsin, sodium dodecyl sulfate (SDS), and glycerol were obtained by Sigma–Aldrich (St. Louis, MO, USA); urea, thiourea, dithiothreitol (DTT), iodoacetamide, tri-*n*-butylphosphate, trifluoroacetic acid, and α-cyano-4-hydroxycinnamic acid were from Fluka (Buchs, Switzerland); Chaps and low-melting agarose were from USB (Cleveland, OH, USA); acetone, methanol, and acetonitrile were from Baker (Deventer, The Netherlands); protease inhibitor cocktail tablets were from Roche (Basel, Switzerland); Immobiline DryStrip 7-cm pH 4–7 gels, IPG buffer pH 3–10, Triton X-100, ECL-Plus, and Percoll were purchased from GE Healthcare (Little Chalfont, UK); and 40% acrylamide: bis solution, 37.5:1, was from Bio-Rad (Hercules, CA, USA).

### *Mouse strains and design of the study*

C57B6/2 J mice (wild-type controls, WT) and two mouse strains that underexpress β-globin chains, the $Hbb^{th/th}$ mouse and the $Hbb^{th3/+}$ mouse, were employed as models of β-thalassemia [20,23]. Male and female mice of 2 to 6 months age (20–25 g body wt) were used. Blood was collected by retro-orbital venipuncture in anesthetized mice using heparinized microcapillary tubes.

### *Measurements of hematological parameters, methemoglobin levels, and hemichromes bound to the membrane*

Blood was centrifuged at 2500 *g* at 4 °C to remove plasma, passed through cotton to eliminate white cells, and washed three times with choline wash solution (180 mM choline, 1 mM $MgCl_2$, 10 mM Tris–Mops, pH 7.4 at 4 °C, 320–340 mOsm). Hematological parameters were evaluated on a Bayer Technicon Analyser ADVIA. Hematocrit and hemoglobin were manually determined [20,23,24]. Red cell MetHb levels were determined as described by Kohn et al. [25]. Measurement of methemoglobin concentration was based on the absorbance of methemoglobin at 630 nm, which is characterized by $\varepsilon_{mM\ 630} = 4.4\ mM^{-1}\ cm^{-1}$ [26]. Addition of cyanide eliminates the contribution of methemoglobin to the absorbance at 630 nm. The absorbance in the absence of cyanide minus that in the presence of cyanide is a measure of the conversion of methemoglobin in the sample to cyanomethemoglobin [25,26].

Hemichromes bound to the membrane were measured as previously described by Ayi et al. [27].

### *Molecular analysis of mouse spleens and bone marrow*

Total RNA was isolated from spleen and bone marrow from wild-type and both of the β-thalassemic mouse models ($n = 4$ from each strain) using Trizol reagent (Invitrogen) according to the manufacturer's protocol. After measurement of RNA yield and quality by NanoDrop machine (Celbio), cDNA was synthesized by random hexamers with the iScript cDNA synthesis kit (Bio-Rad), according to the protocols supplied by Bio-Rad. Two micrograms of total RNA in 20 μl was used in each reaction. QRT-PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems) and the Applied Biosystems Model 7900HT sequence detection system, according to the protocols supplied by Applied Biosystems. Primers were designed with the Primer Express 2.1 program (Applied Biosystems). All of the primers were designed in two different exons to avoid the amplification of genomic DNA. All QRT-PCRs were performed in duplicate. A portion (1.5 μl) of the ss-cDNA synthesis reaction was used in each 25-μl reaction. The α-globin gene (Hba) mRNA was used to normalize the mRNA concentration. The primer sequences for the tested genes were the following: mPrdx2 forward, 5′-CGCCTAGTC-CAGGCCTTTC-3′; mPrdx2 reverse, 5′-GATGGTGTCACTGCCGGG-3′; mHba forward, 5′-TGCGTGTGGATCCCGTC-3′; mHba reverse, 5′-TGAAATCGGCAGGGTGGT-3′.

The relative gene expressions were calculated using the $2^{-\Delta Ct}$ method. The $\Delta C_t$ was calculated using the differences in the mean $C_t$ between the genes and their internal controls [28].

### *Treatment of red cells with oxidative agents*

Red cells from wild-type mice underwent treatment with three different oxidative agents, hydrogen peroxide ($H_2O_2$; 75, 100 μM), diamide (2 mM), and PHZ (20, 50, 100, 250, 500, 750, 1000 μM), in vitro, as we previously reported [15,29]. The concentration of $H_2O_2$ was adapted so that the ratio $H_2O_2$/red cells would be similar to the conditions previously described by Low et al. [30]. Whenever indicated, red cells were pretreated with sodium azide ($NaN_3$; 100 mM) to inhibit catalase before exposure to oxidative agents [1,2].

### *Crossing experiments*

To evaluate whether Prx2 from untreated red cells and from red cells exposed to oxidative stress possesses different degrees of affinity for native or oxidized membrane, we incubated native and oxidatively treated red cell ghosts with cytoplasmic fractions of untreated and oxidatively treated red cells as we previously reported, with minor modifications [31]. We also treated isolated red cell ghosts with either 2 mM diamide or 50 μM phenylhydrazine incubated 10 min at 4 °C; the treated isolated ghosts were then washed three times in 5 mM sodium phosphate, pH 8, 1 mM DTT in the presence of protease inhibitor cocktail and centrifuged at 13,000 *g*, at 4 °C for 10 min.

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