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Peroxiredoxin II is essential for preventing hemolytic anemia from oxidative stress through maintaining hemoglobin stability

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ABSTRACT

The pathophysiology of oxidative hemolytic anemia is closely associated with hemoglobin (Hb) stability; however, the mechanism of how Hb maintains its stability under oxidative stress conditions of red blood cells (RBCs) carrying high levels of oxygen is unknown. Here, we investigated the potential role of peroxiredoxin II (Prx II) in preventing Hb aggregation induced by reactive oxygen species (ROS) using Prx II knockout mice and RBCs of patients with hemolytic anemia. Upon oxidative stress, ROS and Heinz body formation were significantly increased in Prx II knockout RBCs compared to wild-type (WT), which ultimately accelerated the accumulation of hemosiderin and heme-oxygenase 1 in the Prx II knock-out livers. In addition, ROS-dependent Hb aggregation was significantly increased in Prx II knockout RBCs. Interestingly, Prx II interacted with Hb in mouse RBCs, and their interaction, in particular, was severely impaired in RBCs of patients with thalassemia (THAL) and sickle cell anemia (SCA). Hb was bound to the decameric structure of Prx II, by which Hb was protected from oxidative stress. These findings suggest that Prx II plays an important role in preventing hemolytic anemia from oxidative stress by binding to Hb as a decameric structure to stabilize it.

1. Introduction

Peroxiredoxins (Prxs), a family of thiol-containing peroxidases, were identified primarily by their peroxidase activities, and contribute to the control of endogenously produced peroxides in eukaryotes [1]. In addition to their antioxidant activity, Prxs have been implicated in numerous cellular functions, such as proliferation, differentiation [2] and intracellular signaling [3].

Erythrocytes contain a large amount of Hb, which delivers oxygen to all tissues and organs in the body. During the oxygen transport, Hb undergoes autoxidation to produce superoxide [4], which is dismutated to hydrogen peroxide by superoxide dismutase (SOD). Hydrogen peroxide is known to be detoxified by antioxidant enzymes, such as catalase, glutathione peroxidase and Prxs [5]. Prx II is a member of the Prx family that is abundantly expressed in all types of cells. Especially, Prx II is the third most abundant protein and thought to be one of the main players for protection of RBCs from oxidative stress through hemoglobin autoxidation [4]. We have reported that Prx II knockout mice showed Heinz body formation and oxidative hemolytic anemia [6]. Therefore, the redox balance regulated by Prx II in RBCs may be expected to be associated closely with hematological pathologies, such as decreased RBC life span and Hb instability. In addition to the peroxidase function, yeast and human Prxs containing 2-cysteine residues have been shown to act as molecular chaperones under increased oxidative stress [7]. Therefore, current research has been focused on investigating their protection activity in relation to Hb stability and its underlying molecular mechanisms.

In this study, we examined the function of Prx II in the protection of Hb stability in Prx II knockout mice and patients with

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hereditary hemolytic anemia. Our results show that loss of Prx II in mouse RBCs resulted in an elevated ROS level and protein, mainly Hb, aggregation and hypersensitive response to increased ROS. In addition, we also show impaired binding of Prx II to Hb in patients with THAL and SCA RBCs, resulting in the significant increase in the Hb aggregation by ROS attack. These effects were closely associated with a Hb-Prx II interaction. Our findings indicate that the decameric form of Prx II can bind to Hb and protect Hb from oxidative-induced denaturation and aggregation in human and mouse RBCs.

2. Materials and methods

2.1. Mice and patients

Mice of the 129/SvJ background were maintained in a specific pathogen-free authorized facility in the Korean Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon, Korea). All animal procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee, KRIBB.

Peripheral blood were transported in a box with dry ice by using airplane from Israel to Korea and stored at −70 °C until use. Informed consent was obtained in all cases according to the institutional Helsinki Committee regulations.

2.2. Co-immunoprecipitation

The protein samples were incubated with agarose beads (Santa Cruz) for 30 min at 4 °C to pre-absorb any polypeptides that might bind nonspecifically to the beads as described previously [8]. After removing the beads by centrifugation, the supernatant was incubated with anti-Prx II (Lab Frontier, Korea) and Hb antibodies (Santa Cruz) for 1 h and subsequently with protein A-linked agarose beads for 1 h at 4 °C. Proteins were separated by 12% or 15% SDS–PAGE.

2.3. Assay for ROS-induced Hb aggregation

Peripheral blood RBCs (6×10^6) were lysed in 5 mM of phosphate buffer (PB; pH8.0) under native conditions, and an Hb solution was obtained as described previously [9]. Hb was treated with several concentrations of hydrogen peroxide (H_2O_2) for 30 min at 37 °C in the presence or absence of Prx II proteins. Turbidity due to Hb aggregation was analyzed by spectrophotometry (Nano Drop Technologies) at 360 nm as described previously [7].

2.4. Preparation of purified human hemoglobin (phHb)

Human hemoglobin 0.5 g (Sigma Aldrich) was dissolved in 5 ml of loading buffer (20 mM Tris–HCl, pH 8.5, 10 mM NaCl, 5 mM β-mercaptoethanol, 2% polyethylene glycol 4000 (PEG 4K). The Hb solution was placed onto a HiTrap Q FF Column (5 × 1 ml, GE Healthcare) and eluted by elution buffer (20 mM Tris–HCl, pH 8.5, 1 M NaCl, 5 mM β-mercaptoethanol, 2% PEG 4 K). The eluted Hb solution was further purified by dialysis (30 mM Tris–HCl, pH 7.5, 10 mM NaCl, 2 mM β-mercaptoethanol).

2.5. Preparation of recombinant WT and mutant Prx II proteins

The human Prx II (hPrx II) gene was cloned from a human liver library by PCR. N and C terminally truncated hPrx II mutants lacking 14 and 26 amino acids (ΔN-ter and ΔC-ter, respectively) and mutants where the cysteines at positions 51 and 172 were replaced by serine residues. Mutants (C51S and C172S, respectively) were generated by standard PCR-mediated site-directed mutagenesis with pPROEX HTb (Invitrogen). WT and 4 kinds of mutant hPrx II proteins were expressed in *Escherichia coli* BL21 (DE3) and purified using a Ni–NTA column (1 × 4 cm, Ni–NTA superflow; Qiagen), and then cleaved by TEV. Obtained WT and 4 kinds of mutant hPrx II proteins were purified as described previously [10]. To examine the oligomerization states of the WT hPrx II proteins (dimer, decamer, and high molecular form), the WT Prx II proteins were analyzed by size exclusion chromatography (SEC), SEC on HPLC (Dionex) was performed with a Superdex 200 10/30 GL column (GE Healthcare) equilibrated at a flow rate of 0.5 ml/min at 25 °C with a 50 mM HEPES pH 7.0 buffer containing 100 mM NaCl, and the apparent molecular weight of WT hPrx II were confirmed by native-PAGE as described previously [11].

2.6. Statistical analyses

Statistical analysis was performed using ANOVA test. A P value of less than 0.05 was considered to be significant.

3. Results

3.1. Prx II-deficient RBCs fail to resist oxidant-induced Hb aggregation

RBCs are known to require a potent antioxidative defense system. We previously showed that loss of Prx II causes oxidative hemolytic anemia, as evidenced by Heinz body formation in peripheral RBCs and splenomegaly [6]. To further understand the essential role of the Prx II in RBC redox balance, in the present study, we examined the possible connection between ROS level and Hb aggregation rate in Prx II$^{-/-}$ RBCs in response to *in vivo* treatments by aniline hydrochloride (AH) respectively. Prx II$^{-/-}$ RBCs showed higher ROS levels than WT RBCs in response to *in vivo* treatment with AH which reflects oxidative injury in RBCs and induces Heinz body formation [12] (Fig. 1A). In Prx II$^{-/-}$ mice, this event was accompanied by a decrease in hematological parameters, such as hematocrit and Hb content, and increased reticulocyte count in Prx II$^{-/-}$ mice (Table 1). The incidence of Heinz body-containing RBCs had a higher relative increment in Prx II$^{-/-}$ mice (Fig. 1B). These results suggest that the role of Prx II is closely associated with protection of RBCs from ROS-induced Hb aggregation.

3.2. Prx II interacts with Hb and prevents oxidative Hb aggregation

To examine whether Prx II is involved in the protection of Hb against ROS, we prepared cytosolic Hb solution from WT and Prx II$^{-/-}$ RBCs under native conditions, treated them with various concentrations of H_2O_2 in the presence or absence of recombinant human Prx II (rhPrx II), and analyzed Hb aggregation spectrophotometrically (Fig. 2A). Both Hb extracts were aggregated by treatment with H_2O_2, but Prx II$^{-/-}$ Hb were hypersensitive to H_2O_2-induced aggregation compared to WT Hb. However, the addition of rhPrx II restored the aggregation level of Prx II$^{-/-}$ Hb to the level of WT Hb (Fig. 2A). To investigate the underlying mechanism, first we studied the interaction of Prx II with Hb under native conditions using co-immunoprecipitation and Western Blot analyses (Fig. 2B). RBC protein lysates prepared from WT and Prx II$^{-/-}$ RBCs were immunoprecipitated and immunoblotted with anti-Prx II and Hb antibodies. Unlike the result from Prx II$^{-/-}$ RBCs, intense immunoreactive bands against α-Hb and β-Hb were found in WT cytosolic proteins precipitated by the anti-Prx II antibody. Consistent with the result, the Prx II immunoreactive band was only found in proteins from WT cytosolic proteins precipitated by antibodies for α-Hb and β-Hb. These results suggest that Prx II is a novel interacting partner to Hb in RBCs, the interaction of these two proteins