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Peroxiredoxin I deficiency attenuates phagocytic capacity of macrophage in clearance of the red blood cells damaged by oxidative stress

Ying-Hao Han[1,8,#], Taeho Kwon[1,7,#], Sun-Uk Kim[1,2,#], Hye-Lin Ha[1], Tae-Hoon Lee[1,3], Jin-Man Kim[4], Eun-Kyeong Jo[5], Bo Yeon Kim[6], Do Young Yoon[7] & Dae-Yeul Yu[1,]*

[1]Aging Research Center, [2]National Primate Research Center Korea, KRIBB, Daejeon 305-806, [3]Department of Biochemistry, Dental Science Research Institute, Chonnam National University, Gwangju 500-757, Departments of [4]Pathology, [5]Microbiology, College of Medicine, Chungnam National University, Daejeon 301-747, [6]Chemical Biology Research Center and World Class Institute, Korea Research Institute of Bioscience and Biotechnology, Cheongwon 363-883, [7]Department of Bioscience and Biotechnology, Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, Korea, [8]College of Life Science and Technology, Heilongjiang Bayi Agricultural University DaQing, HeiLongJiang province 163319, China

The role of peroxiredoxin (Prx) I as an erythrocyte antioxidant defense in red blood cells (RBCs) is controversial. Here we investigated the function of Prx I by using Prx $I^{-/-}$ and Prx $I/II^{-/-}$ mice. Prx $I^{-/-}$ mice exhibited a normal blood profile. However, Prx $I/II^{-/-}$ mice showed more significantly increased Heinz body formation as compared with Prx $II^{-/-}$ mice. The clearance rate of Heinz body-containing RBCs in Prx $I^{-/-}$ mice decreased significantly through the treatment of aniline hydrochloride (AH) compared with wild-type mice. Prx I deficiency decreased the phagocytic capacity of macrophage in clearing Heinz body-containing RBCs. Our data demonstrate that Prx I deficiency did not cause hemolytic anemia, but showed that further increased hemolytic anemia symptoms in Prx $II^{-/-}$ mice by attenuating phagocytic capacity of macrophage in oxidative stress damaged RBCs, suggesting a novel role of Prx I in phagocytosis of macrophage. [BMB Reports 2012; 45(10): 560-564]

INTRODUCTION

Red blood cells (RBCs) are more exposed to oxidative stress than other cell types due to an abundance of heme iron and oxygen, which can generate H_2O_2 and lipid peroxides. The hydroperoxides themselves are not very reactive but are readily converted in the presence of free iron and electron donor molecules to hydroxyl and alkoxyl radicals, which indiscriminately inflict damage on biomolecules. Therefore, RBCs are believed to have a very effective defense mechanism against peroxides.

Proxiredoxin (Prx) II, a member of peroxiredoxin family, reduces hydrogen peroxide and alkyl hydroperoxides, and controls peroxide levels (1-3), plays a major role in protecting RBCs from oxidative stress in mice (4). We already reported that Prx II knockout mice expressed Heinz body formation and hemolytic anemia although they were healthy in appearance and fertile (4). Heinz bodies, a typical sign of oxidative hemolytic anemia, are inclusions in RBCs composed mainly of denatured hemoglobin (Hb) that caused during blood circulation and prolonged oxidant imbalance (5). Prx I shares 91% homology with Prx II. It is also cytosolic, has the same catalytic mechanism and similar reaction kinetics (6). Many studies have demonstrated that Prx I functions as a cellular regulator in hydrogen peroxide signaling (1, 7), and could protect against hydrogen peroxide mediated cell death (8, 9). Recent studies showed that inactivation of Prx I by phosphorylation allows localized H_2O_2 to accumulate for cell signaling (10) and controls neuronal differentiation by thiol-redox-dependent activation of glycerophosphodiester phosphodiesterase 2 (GDE2) (11). Moreover, Prx I could bind to several proteins. Cao *et al*. found that the binding of peroxidase Prx I to phosphatase and tensin homolog deleted on chromosome ten (PTEN) is essential for protecting PTEN from oxidation-induced inactivation (12). It was reported that Prx $I^{-/-}$ mice expressed hemolytic anemia over 9 months and developed cancer (13). Another group showed that Prx $I^{-/-}$ mice developed age-dependent hemolytic anemia and malignancies (14). However, Uwayama *et al*. (2006) could not find hemolytic anemia symptoms in their Prx $I^{-/-}$ mice and pointed out that the pathogenesis of severe anemia observed in aged Prx $I^{-/-}$ mice studied by Neumann *et al*. is less clear and should be carefully investigated (13, 15).

In the current study, we investigated Prx $I^{-/-}$and Prx $I/II^{-/-}$ mice to more clearly understand the role of Prx I in prevention

*Corresponding author. Tel: +82-42-860-4422; Fax: +82-42-860-4609; E-mail: dyyu10@kribb.re.kr
#These authors equally contributed to this work.
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of hemolytic anemia. Our data demonstrate that Prx $I^{-/-}$ mice showed no phenotype of hemolytic anemia, but exhibited decreased phagocytic capacity for Heinz body-containing RBCs in their macrophages, suggesting a role of Prx I in the regulation of phagocytosis.

RESULTS

Prx I knockout mice showed no sign of Heinz body hemolytic anemia

Prx $I^{-/-}$ mice were generated by replacing all the exons with the neo gene (Fig. 1). We examined the hematological parameters of wild-type and Prx $I^{-/-}$ mice. As shown in Table 1, blood parameters were similar between wild-type and Prx $I^{-/-}$ mice regardless of age. Heinz-body formation in peripheral blood also was not different between two kinds of mice over 10 months (Data not shown), indicating that the Prx $I^{-/-}$ mice generated in our group have no sign of Heinz-body hemolytic anemia.

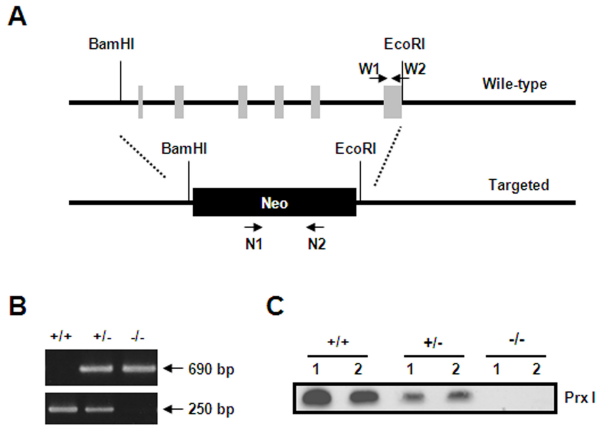

Fig. 1. Targeted disruption of the mouse Prx I gene. (A) Endogenous Prx I gene and the targeting vector are illustrated in the upper and lower part, respectively. The gray boxes indicate Prx I exons and the black box depict a neo gene replaced by homologous recombination. The arrows indicate the four PCR primers used for detection of wild-type allele (W1 and W2) and mutant allele (N1 and N2). (B) Genomic DNA was extracted from the tails of mice and genotyped by the primers indicated in (A). The sizes of PCR bands specific for wild allele and mutant allele are 250 bp and 690 bp, respectively. The genotypes were identified by the combinational appearance of the specific bands. (C) RBCs were collected from two different mice of the indicated genotypes and subjected to Western blotting analysis with antibodies for Prx I.

Heinz body formation in peripheral blood was significantly increased in Prx I/II$^{-/-}$ mice

To know the potential role of Prx I in Heinz-body hemolytic anemia, we generated Prx I/II$^{-/-}$ mice as described in the Material and Methods section, and the genotype was confirmed by the method of polymerase chain reaction (data not shown). Surprisingly, most Prx I/II$^{-/-}$ mice died within 48 hr without a clear reason. But very few of the Prx I/II$^{-/-}$ mice survived and showed higher levels of Heinz-body formation in their peripheral blood than Prx II$^{-/-}$ mice (Fig. 2A). Also, ROS levels were significantly increased in Prx I/II$^{-/-}$ RBCs as compared with Prx II$^{-/-}$ RBCs (Fig. 2B). Consistent with this, histological studies showed that heme oxygenase-1 (HO-1) and hemosiderin were more abundant in the liver of Prx I/II$^{-/-}$ mice than in Prx II$^{-/-}$ mice (Fig. 2C, upper and middle panel). Moreover, F4/80 positive cells were significantly increased in Prx I/II$^{-/-}$ mice compared with other mice (Fig. 2C, lower panel). These results suggest that although Prx I deficiency does not cause Heinz-body hemolytic anemia, it has a synergistic effect in Prx II$^{-/-}$ mice, increasing their Heinz-body formation.

Prx I deficiency decreases clearance of Heinz body-containing RBCs from peripheral blood

To study the role of Prx I in Heinz-body hemolytic anemia, we treated wild-type and Prx $I^{-/-}$ mice with aniline hydrochloride (AH) chronically (1). After treatment, Heinz-body formation was gradually increased in the peripheral blood types of Prx $I^{-/-}$ and wild-type mice, but the number of Heinz body was not significantly different from each other (Fig. 3A). However, clearance of Heinz-body-containing RBCs were remarkably slower in Prx $I^{-/-}$ mice as compared to wild-type mice (Fig. 3A), and the ROS level was higher in Prx $I^{-/-}$ mice (Fig. 3B).

Usually, damaged RBCs are cleared by macrophages in the spleen. We prepared splenic macrophage from wild-type and Prx $I^{-/-}$ mice, and measured the phagocytosis of macrophage for Heinz body-containing RBCs *in vitro*. As shown in Fig. 3C and D, the phagocytic capacity was significantly decreased in Prx $I^{-/-}$ macrophage compared with wild-type mice (Fig. 3C,

Table 1. Hematologic parameters of Prx $I^{-/-}$ mice

Genotypes	Age (Month)	WBC (10^3/μl)	RBC (10^6/μl)	Hb (G/dl)	HCT (%)	MCV (fL)	Retics (%)
+/+	8	7.2 ± 2.4	10.8 ± 0.2	16.3 ± 0.6	53.8 ± 2.4	49.9 ± 1.5	2.6 ± 0.4
−/−	8	6.3 ± 1.6	10.2 ± 0.5	15.9 ± 0.5	52.5 ± 1.9	51.4 ± 1.8	2.1 ± 0.7
−/−	11	6.7 ± 1.2	10.5 ± 0.7	16.1 ± 0.6	51.9 ± 3.0	49.5 ± 0.6	2.4 ± 0.3
−/−	16	7.4 ± 0.7	10.8 ± 0.7	16.1 ± 1.0	53.0 ± 2.6	49.0 ± 0.8	2.6 ± 0.5

WBC: white blood cells, RBC: red blood cells, Hb: hemoglobin, HCT: hematocrit, MCV: mean cell volume, Retics: reticulocytes. These results were obtained from at least 6 mice.

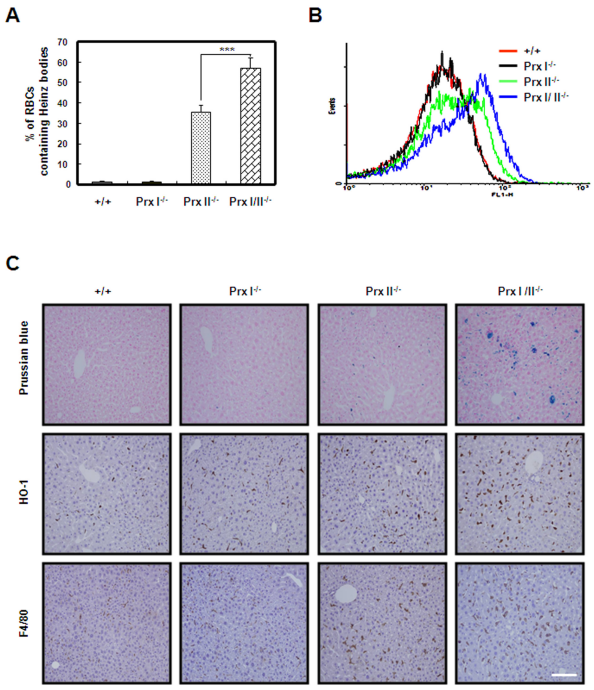

Fig. 2. Synergistic effect of Prx I and Prx II deletions on Heinz-body formation and ROS generation. (A) RBCs were collected from wild-type (+/+), Prx $I^{-/-}$, Prx $II^{-/-}$ and Prx $I/II^{-/-}$ mice (20 weeks), stained with brilliant cresyl blue, and counted under 1,000× magnification using an Olympus microscope. (B) RBCs from four indicated genotypes of mice were stained with DCF-DA and analyzed by FACS. (C) Iron deposition, HO-1 expression and F4/80 positive cells in liver sections of the indicated genotypes were determined by staining with Prussian Blue (upper) and HO-1 or F4/80 antibody by immunohistochemistry (middle and lower), respectively. The scale bar is 50 μm.

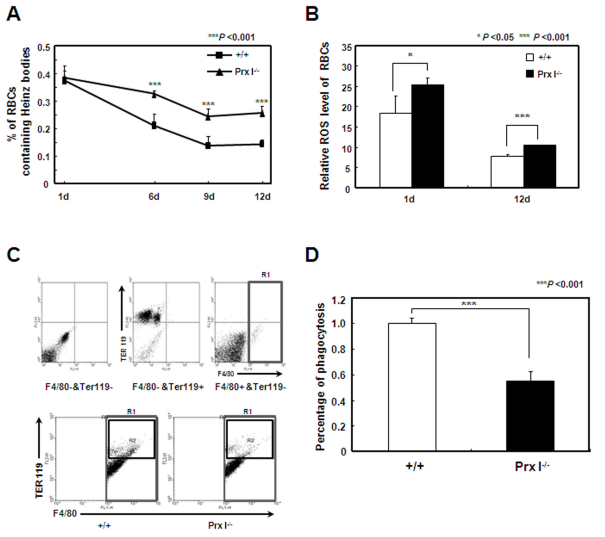

Fig. 3. Clearance of Heinz-body-containing RBCs by macrophage (A) Heinz bodies in peripheral blood of wild-type (+/+) and Prx $I^{-/-}$ mice after injected AH were checked at day 1, 6, 9, and 12. (B) Relative ROS levels were measured at 1 and 12 days for DCF fluorescence intensity by FACS after AH treatment. (C) A Heinz-body-containing RBCs and splenic macrophage were stained with PE-TER119 and FITC-F4/80 antibodies respectively (upper left, middle and right panel). The macrophage-engulfed RBCs were shifted to the R2 region (double positive). Region gate R1 (boxed with grey line in lower panels) represents all F4/80+ cells, whereas region gate R2 (boxed with black line in R1) represents double positive (F4/80+&TER 119+) cells. (D) The phagocytosis of splenic macrophage in wild-type and Prx $I^{-/-}$ mice are shown. The percentages of phagocytosis are derived from the R2 region of wild-type (left) and Prx $I^{-/-}$ (right) mice in Fig3C. Data represent Mean ± SD (n = 3). *P < 0.05, ***P < 0.001.

D). These results indicate that Prx I deficiency decreased phagocytic capacity of macrophages for Heinz-body-containing RBCs, thereby attenuated the removal rate of damaged RBCs from peripheral blood.

DISCUSSION

Heinz bodies are the inclusions in RBCs composed mainly of denatured hemoglobin (Hb). During blood circulation, prolonged oxidant imbalance causes Hb denaturation, resulting in the formation of Heinz bodies, a typical sign of oxidative hemolytic anemia (5). Following oxygen delivery of Hb, superoxide was produced by Hb autoxidation, which was converted to hydrogen peroxide by superoxide dismutase (SOD) and detoxified by antioxidant enzymes, such as glutathione, catalase, glutathione peroxidase, and Prxs. However, catalase, Cu/ZnSOD, or glutathione peroxidase 1 knockout mice did not show Heinz-body formation (16-18). In comparison with this, Prx $II^{-/-}$ mice showed Heinz-body formation and oxidative hemolytic anemia (4). Our study showed that Prx $I^{-/-}$ mice did not show any hemolytic anemia symptoms including Heinz-body formation over 20 months. In contrast with the Prx II, the quantity of Prx I present in erythrocytes was much lower than for Prx II (data not shown) (19). Recent studies have reported that Prx II expression levels in Prx $I^{-/-}$ RBCs appeared normal compared with wild-type mice (15). Uwayama *et al.* (2006) could not find hemolytic anemia symptoms in their Prx $I^{-/-}$ mice up to 35 weeks of age (15). In addition, they have not detected anemia in aged Prx $I^{-/-}$ mice over 1 year (personal communication with Professor Tetsuro Ishii). Thus, we considered that severe hemolytic anemia in the study of aged Prx $I^{-/-}$ mice by Neumann *et al.* (13) and age-dependent hemolytic anemia and malignancies in Prx $I^{-/-}$ mice (14) might be due to the different knockout strategy compared with that we used. They used a retroviral insertional mutagenesis method in generating the knockout mice, whereas we used conventional knockout strategy, replacement method.

Although Prx $I^{-/-}$ mice did not express Heinz-body formation, Prx $I/II^{-/-}$ mice showed more serious symptoms of hemolytic anemia such as higher Heinz-body counts, a higher ROS level in RBCs, and more abundant hemosiderin precipitation in the

liver than was shown in Prx II$^{-/-}$ mice. To understand the reason why Prx I/II$^{-/-}$ mice induce more severe hemolytic anemia than Prx II$^{-/-}$ mice, we treated wild-type and Prx I$^{-/-}$ mice with AH for one month to the level of Prx II$^{-/-}$ mice in Heinz-body formation and then observed the clearance of Heinz-body-containing RBCs. The clearance rate of Heinz-body-containing RBCs was significantly decreased in Prx I$^{-/-}$ mice more than in wild-type mice, associated with decreased phagocytic capacity of Prx I$^{-/-}$ macrophage (Fig. 2). The injection of AH in mice was said to elevate the level of ROS in their peripheral blood, and that ROS could be generated during phagocytosis of macrophage (20) and negatively influenced phagocytosis (21). Therefore we suggest that increased ROS in Prx I$^{-/-}$ mice may attenuate the phagocytic capacity of the macrophage and cause more severe hemolytic anemia symptoms in Prx I/II$^{-/-}$ mice than in Prx II$^{-/-}$ mice. Further studies are needed to understand the detailed mechanism.

Prx I, a protein predominantly expressed in macrophages, served as a defense protein in murine peritoneal macrophages and was markedly induced upon exposure to oxidative stresses (22, 23). Prx I has been expected to protect macrophages against oxidative stress, so also named macrophage 23-kDa stress protein (MSP23). Here we show that Prx I deficiency does not cause Heinz-body formation in peripheral blood, but decrease the phagocytic capacity of macrophage for removing Heinz-body-containing RBCs, indicating a novel role of Prx I in regulation of macrophage phagocytosis in mice.

MATERIALS AND METHODS

Generation of Prx I$^{-/-}$ and Prx I/II$^{-/-}$ mice

Prx I knockout mice were generated by the same method described previously (4). The sequence from exon 1 to exon 6 of a Prx I gene was replaced with the neo gene and the homologous recombination was confirmed by polymerase chain reaction (PCR). The primers used were 5'-CTGGAAACCTGGCA GTGATA-3' and 5'-CTGTGACTGATAGAAGATTGGT-3' for the Prx I gene and 5'-GCT TGG GTG GAG AGG CTA TTC G-3' AND 5'-GTA AAG CAC GAG GAA GCG GTC AGC-3' for the neo gene. Prx I/II$^{-/-}$ mice were obtained by crossing the Prx I$^{-/-}$ mice with previously generated Prx II$^{-/-}$ mice (4), Namely, Prx I$^{-/-}$ and Prx II$^{-/-}$ mice were mated with each other to generate Prx I and Prx II double heterozygous mice, and then these double heterozygous mice were mated each other to produce the Prx I/II$^{-/-}$ mice. Prx I/II$^{-/-}$ mice were generated by the ratio of 1 : 16.

The genetic background of all the mice used in this study were 129/SvJ and they were maintained in a pathogen-free authorized facility in Korea Research Institute of Bioscience and Biotechnology (KRIBB), where the temperature at 20-22°C, the humidity 50-60% and the 12-h-dark/light cycles were maintained. All animal procedures were conducted in accordance with the guidelines of the institutional Animal Care and Use Committee, KRIBB.

Measurement of reactive oxygen species

The intracellular ROS concentration of RBCs was determined using 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA; Invitrogen, Seoul, Korea). RBCs were incubated with 20 μM of DCF-DA for 15 min at 37°C, and the fluorescence intensity was read using a FACS Calibur Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Immunohistochemistry

Mice tissue sections were pretreated with 3% H_2O_2 in 0.1 M Tris-buffered saline (TBS, pH 7.4) for 30 min to quench endogenous peroxidases. Sections were treated with Protein Block solution (DAKO) for 20 min and incubated with rabbit antibody for 30 min in a humid chamber, then incubated with EnVision antirabbit (DAKO, Houston, USA) polymer for 30 min. Visualization of peroxidase bound in the antibody complex was achieved by treatment with 3,3'-diaminobenzidine (DAB) chromogen substrate solution (DAKO). The sections were examined with an Olympus BX51 microscope (Olympus, Tokyo, Japan) under a bright field. Images were acquired with an Olympus DP 70 camera system (Olympus).

Clearance of Heinz-body-containing RBCs in mice after aniline hydrochloride injection

Wild-type (+/+) and Prx I$^{-/-}$ mice were injected intraperitoneally with 160 mg/kg (body weight) of aniline hydrochloride (AH) twice a week for one month. AH is a compound that causes metHb production, which reflects oxidative injury in RBC and induces Heinz body formation (4). The Heinz bodies in the peripheral blood were checked on day 1, 6, 9, and 12 after finishing one month of injections. RBCs were stained with brilliant cresyl blue, mounted onto clean glass-slides, and counted Heinz body formation 1,000× magnification using Olympus microscope.

Phagocytosis assay

The splenic macrophage from wild-type and Prx I$^{-/-}$ mice were isolated by the method described previously (5). Heinz body-forming RBCs (over 90%) were collected from wild-type mice injected with AH (160 mg/kg) for three consecutive days, and labeled with PE-conjugated TER119 (Biolegend USA) antibody. 1×10^7 labeled RBCs were co-cultured with splenic macrophage for 1h at 37°C in a 5% CO_2 atmosphere, and then washed with Hanks' balanced salt solution 3 times to remove remaining RBCs. Splenic macrophages were harvested and labeled with FITC-conjugated F4/80 (Biolegend USA) antibody, then the fluorescence of PE-TER119 and FITC-F4/80 was analyzed using a FACS Calibur Flow Cytometer.

Statistical analysis

The data shown were obtained from a litter-matched group of mice and are representative of at least three experiments with independent groups of animals.

Statistical analysis was performed using an ANOVA test. A P

value of less than 0.05 was considered significant.

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