Irreversible hyperoxidation of peroxiredoxin 2 is caused by tert-butyl hydroperoxide in human red blood cells

Y.I. Ishida a, M. Takikawa b, T. Suzuki b, M. Nagahama a, Y. Ogasawara b,*

a Department of Molecular and Cellular Biochemistry, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo 204-8588, Japan
b Department of Analytical Biochemistry, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo 204-8588, Japan

ARTICLE INFO

Article history:
Received 31 July 2014
Revised 3 October 2014
Accepted 3 October 2014

Keywords:
Peroxiredoxin 2
Oxidative stress
Red blood cell
tert-Butyl hydroperoxide
Hyperoxidation
Biomarker

ABSTRACT

Peroxiredoxin 2 (Prx2) is the third most abundant protein in red blood cells (RBCs). In this study, we have succeeded in implementing the rapid and simultaneous detection of the hyperoxidized (Prx2-SO2/3) and reduced (Prx2-SH) forms of Prx2 in human RBCs using reverse phase high-performance liquid chromatography. The detection of a peak corresponding to Prx2-SO2/3 was clearly observed following treatment of tert-butyl hydroperoxide (t-BHP), but not H2O2, and was found to be dose-dependent. The identity of the peak was confirmed as Prx2 by immunoblotting and mass spectrometry analysis. Our results suggest that t-BHP hyperoxidizes cysteine residues in Prx2 more readily than H2O2, and that accumulation of hyperoxidized Prx2 might reflect disruption of redox homeostasis in RBCs.

© 2014 The Authors. Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).

1. Introduction

Peroxiredoxins (Prxs) constitute a group of redox enzymes that eliminate hydrogen peroxide using thioredoxin as the substrate; the isoforms Prx1–6 have been identified in recent studies [1]. Prxs contain cysteine residues that are highly sensitive to oxidation by peroxides [2]. It is also known that Prxs react with low level H2O2 at cysteine residues in the active site [2]. In cells, the oxidation state (disulfide form or reduced monomer) of cysteine residues is reversely regulated by the thioredoxin- and sulfiredoxin-dependent reductase systems [3]. However, the contribution of the reductase systems in human red blood cells (RBCs) remains unclear [4,5]. Prxs are antioxidative proteins and Prx2 has been found to be dose-dependent. The identity of the peak was confirmed as Prx2 by immunoblotting and mass spectrometry (MS) techniques.

In this study, we assessed a possible method for detecting oxidation of Prx2, which is an abundant protein in RBCs. The production of hyperoxidized Prx2 in reactions with hydrogen peroxide (H2O2) and tert-butyl hydroperoxide (t-BHP) was investigated to establish a reverse-phase mode high performance liquid chromatography (HPLC)-based procedure for the separation and UV detection of the reduced and hyperoxidized forms of Prx2. Further, we conducted a more detailed analysis focusing on the oxidation state of susceptible thiol residues in hyperoxidized Prx2 produced by the treatment of human RBCs with t-BHP using specific antibody and mass spectrometry (MS) techniques.

2. Materials and methods

2.1. Chemicals

RIPA solution, tert-butyl hydroperoxide (t-BHP), and 3-amino-1,2,4-triazol (3-AT) were purchased from Sigma Aldrich (St. Louis, MO, USA). Dithiothreitol (DTT), mercaptosuccinate and hydrogen...
peroxide \( (H_2O_2) \) (atomic absorption grade) were obtained from Wako Pure Chemical Industries (Osaka, Japan). The protease inhibitor cocktail was obtained from Thermo Scientific (Waltham, MA, USA). HPLC grade water and acetonitrile were obtained from Nacalai Tesque (Kyoto, Japan). All other reagents were of the highest commercial grade available.

2.2. Preparation of RBCs and lysates

Blood samples were obtained from eight healthy subjects (21–51 years old; fasted for 12 h) with their informed consent. Blood was drawn into vacutainer tubes containing ethylenediamine tetraacetic acid (EDTA). RBCs were prepared by centrifugation at 800 \( \times \) g for 10 min at 4 °C and were washed in phosphate-buffered saline [PBS: 137 mM NaCl, 8.1 mM \( Na_2HPO_4 \), 2.68 mM KCl, 1.47 mM KH\(_2\)PO\(_4\) (pH 7.4)] using the same centrifugation procedure. RBCs were resuspended in PBS (Hct. 0.40) containing 10 mM glucose and 1 mM diethylenetriaminepentaacetic acid (DTPA).

To avoid the artificial oxidation of Prx2 during the direct analysis of the hemolysate by reverse phase HPLC assay, RBCs were lysed in a hypotonic buffer [5 mM phosphate buffer (pH 7.4) containing 0.1% Triton-X 100, protease inhibitors] containing 5 mM DTT after incubation with or without each peroxide. The lysates were centrifuged at 15,000 \( \times \) g for 5 min at 4 °C and the supernatant was used as the sample for analysis of Prx2. All of the hemolysates were prepared within 60 min of collection and were subsequently rapidly separated using HPLC.

2.3. Hyperoxidation of Prx2 in RBCs

Irreversible oxidation of Prx2 was induced by \( H_2O_2 \) or t-BHP treatment. The RBC suspension (hematocrit adjusted to 40%) was incubated at 37 °C with 100–500 \( \mu \)M peroxides for 60 min, after which the RBCs were washed twice in PBS. Following hemolysis of the RBCs in a hypotonic buffer, the hemolysate was centrifuged to remove insoluble debris. The supernatant was immediately applied to a reverse phase HPLC system for protein separation.

2.4. HPLC system

Reverse phase HPLC was performed using a C18 column for protein (YMC-packed PROTEIN-RP; YMC Co., Tokyo, Japan) with a UV detector (280 nm). The RBC lysate was injected into the column, which was equilibrated with 40% acetonitrile, at a flow rate of 1.0 ml/min. HPLC was conducted using two mobile phases, A [water containing 0.1% trifluoroacetic acid (TFA)] and B (acetonitrile containing 0.1% TFA). The elution was conducted according to the following sequence: 40% B (0 min) - 40% B (20 min) - 45% B (50 min) - 90% B (50.1 min) - 90% B (60 min) - 40% B (60.1 min) - 40% B (70 min).

2.5. Western blot analysis and detection of Prx2 and Prx-SO2/3

Equal amounts of protein were subjected to SDS–PAGE (Any KD gel; Bio-Rad, Hercules, CA, USA) with a reducing condition and without a non-reducing condition DTT-reduction. For the immunoblot analyses of Prx2 and hyperoxidized Prx2 (Prx-SO2/3), the proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane; the membranes were blocked with 0.1% (w/v) skim milk containing TPBS [0.1% Tween (v/v) containing PBS (pH 7.4)], and were subsequently washed three times with TPBS. The washed PVDF membranes were then incubated for 1 h at room temperature with anti-Prx2 antibody (monoclonal; Abfrontier, South Korea) or an anti-Prx-SO2/3 antibody (polyclonal; Abfrontier). After extensive washing in TPBS, the blots were incubated at room temperature for 1 h with anti-mouse or anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Vector, Burlingame, CA, USA). The band images were captured using chemiluminescence reagents (Immobilon; Merck Millipore, Darmstadt, Germany) with an ImageQuant 400 (GE Healthcare, Japan Ltd., Tokyo, Japan).

2.6. Two-dimensional SDS–PAGE

Cell lysates or purified proteins were mixed with rehydration buffer (8 M urea, 2% CHAPS, 0.5% immobilized pH gradient buffer, 20 mM DTT, and 0.005% bromophenol blue) and loaded onto immobilized pH gradient strips (pH 4–7, 7 cm, Ready Strip IGC; Bio-Rad). Isoelectric focusing was carried out in four steps as follows: 250 V, 15 min; 4000 V, 1.0 h; 4000 V, 8–10,000 V-h, 500 V, 24 h. After reduction and alkylation, second dimensional electrophoresis was conducted on an SDS–PAGE with a 5–20% gradient gel (ATTO, Tokyo, Japan).

2.7. Protein identification

Identification of the proteins of interest was performed by in-gel digestion and peptide mass fingerprinting (PMF) using MS as described previously, with some modifications [12,13]. Briefly, gels were stained with a Silver Stain MS Kit (Wako Pure Chemical Industries) and the protein bands of interest were excised. The eluted fractions were subjected to SDS–PAGE, followed by Western blotting detection with anti-Prx2 antibodies. Fig. 1A shows a chromatogram of lysates from native RBCs and the separation pattern of Prx2. Using the gradient program that we established in this study, Prx2 was detected at a retention time of 42 min (Peak-a, Fig. 1A). Additionally, a minor band was observed at a retention time of 46 min tended to increase. Following pretreatment with inhibitors (10 mM 3-AT for catalase, 7 mM succinate for GPx1)

3. Results

First, we investigated the separation conditions for Prx2 in RBC lysates using reverse-phase columns for protein separation. The eluted fractions were subjected to SDS–PAGE, followed by Western blotting detection with anti-Prx2 antibodies. Fig. 1A shows a chromatogram of lysates from native RBCs and the separation pattern of Prx2. Using the gradient program that we established in this study, Prx2 was detected at a retention time of 42 min (Peak-a, Fig. 1A). Additionally, a minor band was observed at a retention time of 46 min. We next examined the effect of oxidative stress on Prx2 elution using the commonly employed oxidant \( H_2O_2 \). Compared to control (Fig. 1A), changes in the chromatogram and separation pattern of Prx2 were not prominent in \( H_2O_2 \)-treated RBCs (Fig. 1B), although the low intensity Prx2 band at a retention time of 46 min tended to increase. Following pretreatment with inhibitors (10 mM 3-AT for catalase, 7 mM succinate for GPx1)
before exposure of RBC suspensions to 500 μM H₂O₂, no significant hyperoxidation of Prx2 was observed (data not shown).

In contrast, the intensity of the peak corresponding to native Prx2 (Peak-b, Fig. 1C) decreased following treatment with t-BHP, which is more hydrophobic than H₂O₂. Concomitant with the decrease in native Prx2 (Peak-b), a new peak appeared at a retention time of 46 min (Peak-c, Fig. 1C). Quantitative analysis of the Prx2 peaks showed that these alterations occurred in a t-BHP concentration-dependent manner (Fig. 2). Although it is likely that t-BHP-induced hyperoxidation is more obvious at high concentrations, a low concentration (~100 μM) of t-BHP significantly hyperoxidized Prx2 in human RBCs (Fig. 2).

Second, for a more detailed assessment of the separation capacity of Prx2 in the present HPLC system, protein composition in the Prx2-detected fractions (Peak-a and Peak-c) was evaluated by SDS–PAGE followed by silver staining. As shown in Fig. 3, the most prominent bands detected in both fractions (bands No. 1 and No. 2) were proteins of ~23 kDa. Using in-gel digestion and PMF (MALDI–TOF–TOF-MS), proteins derived from bands No. 1 and No. 2 were both identified as human Prx2 (Accession: UniProtKB:P32119) with sequence coverages of 51.5% and 65.7%, respectively.

To clarify the oxidative status of Prx2, two-dimensional (2D) electrophoresis of the t-BHP-treated sample was performed,
followed by immunoblot detection of Prx2. The results showed two spots of equal molecular weight with \( p_I \) values of 5.8 and 5.4; the more acidic spot (\( p_I 5.4 \)) is more clearly observed in the \( t \)-BHP-treated sample compared to the control sample (Fig. 4). Furthermore, the two peaks obtained from the oxidized samples by reverse phase HPLC were also examined by immunoblotting. While similar band intensities (23 kDa) were observed for both peaks detected with the anti-Prx2 antibody, anti-PrxSO2/3 immunoreactivity was markedly increased in Peak-c compared to Peak-b (Fig. 5). These results indicated that the native and hyperoxidized forms of Prx2 were separated by our HPLC system.

4. Discussion

Prxs are a widespread family of cysteine-dependent peroxidases, and their oxidation state is presumed to be involved in other functions such as chaperone activity [14]. Using antibodies specific for sulfinic and sulfonic acids (Prx-SO2/3), various examples have been found of Prx hyperoxidation in physiologically relevant events. Given the need to better understand the biological roles of Prx hyperoxidation, it is important to be able to characterize this property in the Prx family. We have recently focused our attention on the physiological significance of Prx2, which is abundant in RBCs, and have found that the oligomers consisting of the reduced form of Prx2 in human RBCs exhibit chaperone activity nearly equal to that of the hyperoxidized form [15]. Thus, real-time monitoring of the hyperoxidation status was suggested to be significant for studying the physiological roles of Prxs.

In this study, we developed a reverse-phase HPLC-based method for the rapid separation of reduced and hyperoxidized Prx2 using human RBCs as the sample, in which the activity of the reversible redox system is low. In the present method, the sample preparation and assay procedures were shown to easily separate and detect the reduced and hyperoxidized forms of Prx2. Therefore, this method is more useful than 2D-PAGE with Western blot analysis in yielding accurate results that reflect the hyperoxidative status of human RBCs. Indeed, the present study demonstrated that the detection of Prx2 hyperoxidation and estimation of the protein oxidation state can occur within 2 h after blood sampling. To verify the production and accumulation of the hyperoxidized form of Prx2 in RBCs, we also estimated the oxidative status using 2D electrophoresis/Western blot analysis.

As shown in Fig. 4, significant spots that have maintained the \( p_I \) of native Prx2 were detected with anti-Prx-SO2/3 antibody. It is likely that these spots observed are artificially hyperoxidized proteins produced after the separation by isoelectric focusing followed by PAGE and Western blot analysis, as very little peak corresponding to hyperoxidized Prx2 was observed in the HPLC results for the same untreated sample (Fig. 1B), and a faint band was observed with anti-Prx-SO2/3 antibody for the same \( t \)-BHP treated sample (Fig. 5). Concurrently, good separation between the reduced and hyperoxidized forms of Prx2 was achieved when reversed phase HPLC was utilized to assess Prx2 in RBC samples. The peaks on the chromatogram were identified using MALDI–TOF-MS. Observation of hyperoxidized Prx2, formed upon exposure to cytotoxic ROS, revealed that Prx2 in RBCs might be refractory to oxidation by peroxides, since the hyperoxidized form was produced upon exposure to \( t \)-BHP (100–500 \( \mu \)M) but not to \( H_2O_2 \) (500 \( \mu \)M). In addition to Prx2, protein bands of \( \sim 27 \) kDa and 53 kDa were detected in control and \( t \)-BHP treated fractions, respectively. These proteins are likely to be Prx2-binding proteins whose interaction is affected by the hyperoxidative status of Prx2, although there are other possible explanations, such as accidental coincidence due to equivalent retention times.

Rhee and co-workers provided the first LC–Q–TOF-MS evidence for reversible conversion of cysteine residues of Prx1, 2, and 3 to sulfinic acid (–SO\(_2\)) in a rat macrophage-derived cell line [16,17]. Moreover, they reported that Cys residues in yeast Prx1 are hyperoxidized to sulfonic acid (–SO\(_3\)), based on TOF-MS analysis [18]. Rabilloud et al. reported that a sensitive cysteine, Cys-51, was shown to be oxidized to cysteine sulfonic acid by \( t \)-BHP, leading to the formation of an inactivated form of Prx2 in human T-lymphoma cells [19]. However, there are insufficient studies dealing with the oxidation status of freshly prepared Prx2 from human RBCs, since detailed analysis requires long periods of time for 2D electrophoresis following by Western blot analysis. Our HPLC-based method is not only rapid, but also more useful for quantitation in comparison with Western blot analysis after separation by 2D-PAGE, which provides qualitative rather than quantitative data. Indeed, it is indicated that the antibodies available typically recognize sulfonic acid forms more strongly than native forms [20].
Yang et al. reported that H$_2$O$_2$-hyperoxidized recombinant human Prx1 eluted earlier than native Prx1 from a reverse-phase HPLC system [21]. The hyperoxidized Prx2 in human RBCs treated by t-BHP was retained more strongly by the reverse-phase HPLC column than native Prx2. Thus, we propose that the hydrophobicity is not only dependent on the oxidation state of the Cys-51 residue, but also on the steric conformation of oxidized Prx2. Our results appear to indicate that a form of hyperoxidized Prx2 was altered to become more hydrophobic than native Prx2.

In conclusion, the present study suggests that the oxidation-susceptible Cys residue of Prx2 from human RBC was oxidized into a hyperoxidized (sulfinic and sulfonic acids) derivative by t-BHP. This purification procedure might be applied to real-time assays for detecting reduced and hyperoxidized Prx2. Consequently, this method may enable determination of the oxidative status and redox balance in Prx2 in vivo. Further investigations using this method into the physiological function of Prx2 and its application to clinical specimens are expected in the near future.

Author contributions

Y.O. and M.N. designed and performed research; Y.I., M.T. and T.S. acquired and analyzed data; Y.I. and Y.O. wrote the paper.

Acknowledgement

This work was partially supported by JSPS KAKENHI Grant Number 24590766.

References

[1] Hall, A., Karplus, P.A. and Poole, L.B. (2009) Typical 2-Cys peroxiredoxins – structures, mechanisms and functions. FEBS J. 276, 2469–2477.
[2] Low, F.M., Hampton, M.B. and Winterbourn, C.C. (2008) Peroxiredoxin 2 and peroxide metabolism in the erythrocyte. Antioxid. Redox Signal. 10, 1621–1636.
[3] Rhee, S.G. and Woo, H.A. (2011) Multiple functions of peroxiredoxins: peroxidases, sensors and regulators of the intracellular messenger H$_2$O$_2$ and protein chaperones. Antioxid. Redox Signal. 15, 781–794.
[4] Cha, M.K. and Kim, J.H. (1995) Thioredoxin-linked peroxidase from human red blood cell: evidence for the existence of thioredoxin and thioredoxin reductase in human red blood cell. Biochem. Biophys. Res. Commun. 217, 900–907.
[5] Low, F.M., Hampton, M.B., Peskin, A.V. and Winterbourn, C.C. (2007) Peroxiredoxin 2 functions as a noncatalytic scavenger of low-level hydrogen peroxide in the erythrocyte. Blood 109, 2611–2617.
[6] Poynton, R.A. and Hampton, M.B. (2014) Peroxiredoxins as biomarkers of oxidative stress. Biochim. Biophys. Acta 1840, 906–912.
[7] Matte, A., Low, P.S., Turrini, F., Bertoldi, M., Campanella, M.E., Spano, D., Pantaleo, A., Siciliano, A. and De Franceschi, L. (2010) Peroxiredoxin-2 expression is increased in beta-thalassemic mouse red cells but is displaced from the membrane as a marker of oxidative stress. Free Radic. Biol. Med. 49, 457–466.
[8] Rinalducci, S., D’Amici, G.M., Blasi, B., Vaglio, S., Grazzini, G. and Zolla, I. (2011) Peroxiredoxin-2 as a candidate biomarker to test oxidative stress levels of stored red blood cells under blood bank conditions. Transfusion 51, 1439–1449.
[9] Rinalducci, S., D’Amici, G.M., Blasi, B. and Zolla, I. (2011) Oxidative stress-dependent oligomeric status of erythrocyte peroxiredoxin II (PrxII) during storage under standard blood banking conditions. Biochimie 93, 845–853.
[10] Cumming, R.C., Dargusch, R., Fischer, W.H. and Schubert, D. (2007) Increase in expression levels and resistance to sulfhydryl oxidation of peroxiredoxin isoforms in amyloid beta-resistant nerve cells. J. Biol. Chem. 282, 30523–30534.
[11] Yoshida, Y., Yoshikawa, A., Kinumi, T., Ogawa, Y., Saito, Y., Obara, K., Yamamoto, H., Imai, Y. and Niki, E. (2009) Hydroxyoctadecadienoic acid and oxidatively modified peroxiredoxins in the blood of Alzheimer’s disease patients and their potential as biomarkers. Neurobiol. Aging 30, 174–185.
[12] Ishida, Y.I., Yamazaki, M., Yuki, C., Nishiyama, K., Tsoubouchi, H., Okayama, A. and Kataoka, H. (2014) Carnosol, rosemary ingredient, induces apoptosis in adult T-cell leukemia/lymphoma cells via glutathione depletion: proteomic approach using fluorescent two-dimensional differential gel electrophoresis. Hum. Cell 27, 68–77.
[13] Takeshiba, M., Ishida, Y.I., Akamatsu, E., Ohmori, Y., Sudo, M., Uto, H., Tsoubouchi, H. and Kataoka, H. (2009) Ponoanthocyanidin from blueberry leaves suppresses expression of subgenomic hepatitis C virus RNA. J. Biol. Chem. 284, 21165–21176.
[14] Jang, H.H., Lee, K.O., Chi, Y.H., Jung, B.G., Park, S.K., Park, J.H., Lee, J.R., Lee, S.S., Moon, J.C., Yun, J.W., Choi, Y.O., Kim, W.Y., Kang, J.S., Cheong, G.W., Yun, D.J., Rhee, S.G., Cho, M.J. and Lee, S.Y. (2004) Two enzymes in one; two yeast peroxiredoxins display oxidative stress-dependent switching from a peroxidase to a molecular chaperone function. Cell 117, 625–635.
[15] Ogawara, Y., Ohminato, T., Nakamura, Y. and Ishii, K. (2012) Structural and functional analysis of native peroxiredoxin 2 in human red blood cells. Int. J. Biochem. Cell Biol. 44, 1072–1077.
[16] Woo, H.A., Chae, H.Z., Hwang, S.C., Yang, K.S., Kang, S.W., Kim, K. and Rhee, S.G. (2003) Reversing the inactivation of peroxiredoxins caused by cysteine sulfenic acid formation. Science 300, 653–656.
[17] Woo, H.A., Kang, S.W., Kim, H.K., Yang, K.S., Chae, H.Z. and Rhee, S.G. (2003) Reversible oxidation of the active site cysteine of peroxiredoxins to cysteine sulfenic acid. Immuno blot detection with antibodies specific for the hyperoxidized cysteine-containing sequence. J. Biol. Chem. 278, 47361–47364.
[18] Lim, J.C., Choi, H.I., Park, Y.S., Nam, H.W., Woo, H.A., Kwon, K.S., Kim, Y.S., Rhee, S.G., Kim, K. and Chae, H.Z. (2008) Irreversible oxidation of the active-site cysteine of peroxiredoxin to cysteine sulfenic acid for enhanced molecular chaperone activity. J. Biol. Chem. 283, 28873–28880.
[19] Kabilloud, T., Heller, M., Gasnier, F., Luche, S., Rey, C., Aebischer, R., Benahmed, M., Louissot, P. and Lunardi, J. (2002) Proteomics analysis of cellular response to oxidative stress. Evidence for in vivo overoxidation of peroxiredoxins at their active site. J. Biol. Chem. 277, 19396–19401.
[20] Nelson, K.J., Parsonsge, D., Karplus, P.A. and Poole, L.B. (2013) Evaluating peroxiredoxin sensitivity toward inactivation by peroxide substrates. Methods Enzymol. 527, 21–40.
[21] Yang, K.S., Kang, S.W., Woo, H.A., Hwang, S.C., Chae, H.Z., Kim, K. and Rhee, S.G. (2002) Inactivation of human peroxiredoxin 1 during catalysis as the result of the oxidation of the catalytic site cysteine to cysteine-sulfenic acid. J. Biol. Chem. 277, 38029–38036.