FEBS Open Bio 4 (2014) 848–852

FEBS openbio

journal homepage: www.elsevier.com/locate/febsopenbio

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

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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-$SO_{2/3}$) 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-$SO_{2/3}$ was clearly observed following treatment of *tert*-butyl hydroperoxide (*t*-BHP), but not H_2O_2, 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 H_2O_2, and that accumulation of hyperoxidized Prx2 might reflect disruption of redox homeostasis in RBCs.

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 H_2O_2 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 u nclear [4,5]. Prxs are antioxidative proteins and Prx2 has been the focus of attention as a possible oxidative stress marker [6,7]. Indeed, certain studies have reported that hyperoxidized Prx2 can serve as an indicator of oxidation in blood preservation [8,9]. Additionally, the hyperoxidized forms of Prx family proteins have been found in neuronal cells [10] and the brains of patients with Alzheimer's disease [11]. Although notable and interesting reports focusing on the oxidation of Prx2 are steadily increasing, the oxidation state and physiological formation process of irreversibly oxidized Prx2, termed the "hyperoxidized form", are not well understood in human RBCs.

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 (H_2O_2) 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

Abbreviations: *t*-BHP, *tert*-butyl hydroperoxide; DTPA, diethylenetriaminepentaacetic acid; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; PMF, peptide mass fingerprinting; PBS, phosphate-buffered saline; RBC, red blood cell; ROS, reactive oxygen species; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TOF, time-of-flight; MS, mass spectrometry; TFA, trifluoroacetic acid

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http://dx.doi.org/10.1016/j.fob.2014.10.003

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×*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_2PO_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×*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 μM 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 C_{18} 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-$SO_{2/3}$

Equal amounts of protein were subjected to SDS–PAGE (Any KD gel; Bio-Rad, Hercules, CA, USA) with (reducing condition) or without (non-reducing condition) DTT-reduction. For the immunoblot analyses of Prx2 and hyperoxidized Prx2 (Prx-$SO_{2/3}$), 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-$SO_{2/3}$ 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 IPG; 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 finger printing (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 gel pieces were destained in a 1:1 solution of 100 mM sodium thiosulfate and 30 mM potassium ferricyanide and subsequently incubated in a reducing solution (25 mM NH_4HCO_3 and 25 mM DTT) for 20 min at 56 °C, followed by further incubation for 20 min at room temperature in an alkylation solution (25 mM NH_4HCO_3 and 55 mM iodoacetamide). The gel pieces were dehydrated with acetonitrile and incubated in 20 μl of digestion solution [50 mM NH_4HCO_3, 2 μg/ml trypsin (Trypsin Gold, mass spectrometry grade; Promega, Madison, WI, USA) and 0.01% ProteaseMax (Promega)] for 10 min at room temperature, followed by the addition of 10 μl digestion solution without trypsin. After incubation for 3 h at 42 °C, the peptides produced were extracted with 2.5% TFA and spectra were obtained using matrix-assisted laser desorption/ionization (MALDI)–time of flight (TOF)–TOF-MS (UltrafleXtreme; Bruker Daltonics, Bremen, Germany). The data set was entered into an in-house Mascot search engine (Matrix Sciences, London, UK) to find the closest match with known proteins registered in the Swiss-Prot database.

2.8. Other methods

Protein concentrations were determined using a BCA kit (Thermo Scientific) with bovine serum albumin as a standard.

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)