Archives of Biochemistry and Biophysics 527 (2012) 45–54

Contents lists available at SciVerse ScienceDirect

Archives of Biochemistry and Biophysics

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

Protein thiol oxidation and formation of S-glutathionylated cyclophilin A in cells exposed to chloramines and hypochlorous acid

Melissa M. Stacey [1,2], Sarah L. Cuddihy [1], Mark B. Hampton, Christine C. Winterbourn *

Free Radical Research Group, Department of Pathology and the National Research Centre for Growth and Development, University of Otago Christchurch, P.O. Box 4345, Christchurch, New Zealand

ARTICLE INFO

Article history:
Received 1 June 2012
and in revised form 21 July 2012
Available online 31 July 2012

Keywords:
Chloramine
Hypochlorous acid
Cyclophilin A
Glutathionylation
Oxidation
Peroxiredoxin
Cofilin

ABSTRACT

Neutrophil oxidants, including the myeloperoxidase products, HOCl and chloramines, have been linked to endothelial dysfunction in inflammatory diseases such as atherosclerosis. As they react preferentially with sulfur centers, thiol proteins are likely to be cellular targets. Our objectives were to establish whether there is selective protein oxidation in vascular endothelial cells treated with HOCl or chloramines, and to identify sensitive proteins. Cells were treated with HOCl, glycine chloramine and monochloramine, reversibly oxidized cysteines were labeled and separated by 1D or 2D SDS–PAGE, and proteins were characterized by mass spectrometry. Selective protein oxidation was observed, with chloramines and HOCl causing more changes than H_2O_2. Cyclophilin A was one of the most sensitive targets, particularly with glycine chloramine. Cyclophilin A was also oxidized in Jurkat T cells where its identity was confirmed using a knockout cell line. The product was a mixed disulfide with glutathione, with glutathionylation at Cys-161. Glyceraldehyde-3-phosphate dehydrogenase, peroxiredoxins and cofilin were also highly sensitive to HOCl/chloramines. Cyclophilins are becoming recognized as redox regulatory proteins, and glutathionylation is an important mechanism for redox regulation. Cells lacking Cyclophilin A showed more glutathionylation of other proteins than wild-type cells, suggesting that cyclophilin-regulated deglutathionylation could contribute to redox changes in HOCl/chloramine-exposed cells.

Introduction

Neutrophils are attracted to inflammatory sites where they can be activated to generate superoxide radicals and other reactive oxygen species. Bystander cells are therefore at risk of oxidative injury in inflammatory diseases. The endothelium is particularly vulnerable in vascular inflammation and endothelial dysfunction is an important contributor to the pathology of cardiovascular diseases [1–3]. Neutrophils contain myeloperoxidase, an enzyme that uses the hydrogen peroxide[3] (H_2O_2) generated in the oxidative burst to oxidize chloride to hypochlorous acid (HOCl) [4–7]. Further reaction of HOCl with amine groups (for example in proteins, amino acids or ammonia) produces chloramines (RNHCl). HOCl, and to a lesser extent chloramines, are strong cytotoxic oxidants [8–10]. Cells exposed to low concentrations of HOCl and cell-permeable chloramines show increased membrane permeability and potassium loss [11,12]. These oxidants have also been shown to inhibit oxygen uptake and glucose metabolism and cause loss of ATP and NAD [11,13], activate MAP kinase pathways and initiate growth arrest [14–16], influence NFκB-dependent cytokine and adhesion molecule expression [17], affect NO metabolism [18,19], and induce apoptosis [8].

HOCl and chloramines react preferentially with sulfur centers [20,21], and it is likely that many of their effects on cells are mediated by thiol oxidation. Glutathione (GSH) [22] and individual thiol proteins have been shown to be sensitive to these oxidants, either in cells or in isolation. For example, oxidation of cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [11,22,23], plasma membrane ATPases [11], creatine kinase [24], cofilin [25] and α-ketoglutarate dehydrogenase [26] has been described. However, there is less information on the relative sensitivities of these proteins to oxidation by reactive chlorine species. Klamt et al. [25] carried out a study with the chloramine derivative of taurine, in which they detected reversible oxidation of several thiol proteins. However, taurine chloramine has very low cell permeability (and

* Corresponding author. Fax: +64 3 364 1083.
E-mail addresses: stacey@lunenfeld.ca (M.M. Stacey), sarah.cuddihy@otago.ac.nz (S.L. Cuddihy), mark.hampton@otago.ac.nz (M.B. Hampton), christine.winterbourn@otago.ac.nz (C.C. Winterbourn).

[1] These authors contributed equally to the study.

[2] Present address: Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada M5G 1X5.

[3] *Abbreviations used:* H_2O_2, hydrogen peroxide; GAPDH, glyceraldehyde-3 dehydrogenase; GlyCl, glycine chloramine; NH_2Cl, monochloramine; IAF,5-iodoacetamidofluorescein; HUVEC, human umbilical vein endothelial cells; CypA, Cyclophilin A; tris, tris(hydroxymethyl)aminomethane; 2DE, 2-dimensional gel electrophoresis.

0003-9861/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.abb.2012.07.011

scavenging by taurine can protect intracellular targets against oxidation by HOCl [23,27]), and oxidative changes were seen after extended incubation.

Our aim was to use a redox proteomic approach to identify proteins that are sensitive to oxidation by HOCl and cell permeable chloramines and to compare the pattern of changes with those caused by H_2O_2. We treated human umbilical vein endothelial cells (HUVEC) with HOCl, glycine chloramine (GlyCl) and monochloramine (NH_2Cl) and detected reversibly oxidized thiol proteins by adding N-ethylmaleimide to the cells to block all free thiols, reducing the oxidized thiols and labeling the released SH groups with a fluorescent tag (5-iodoacetamidofluorescein; IAF). A number of oxidative changes were detected by 1D or 2D electrophoresis with protein identification by mass spectrometry. Cyclophilin A (CypA) was identified as being particularly sensitive to oxidation in chloramine-treated HUVECs; these findings were reproduced in Jurkat cells and we furthermore established that this protein undergoes glutathionylation on its C-terminal cysteine.

Materials and methods

Materials

IAF, cell culture media and supplies were from Invitrogen (Carlsbad, CA, USA). HyClone New Zealand Cosmic Calf Serum was from Global Science & Technology Ltd., (Auckland, NZ). Complete™ protease inhibitors, tris(hydroxymethyl)aminomethane (tris) and CHAPS were from Roche (Indianapolis, IN, USA). Anti-CypA antibody was from Santa Cruz (Santa Cruz, CA, USA), anti-GSH monoclonal antibody from Virogen (Watertown, MA, USA), anti-mouse HRP from GE Healthcare (Buckinghamshire, UK), anti-Prx1 from Abcam (Cambridge, UK), anti-GAPDH, anti-Prx3 and anti-$PrxSO_{2/3}$ from AbFrontier (Seoul, South Korea) and anti-Prx2, anti-actin monoclonal antibody and anti-rabbit HRP from Sigma (St. Louis, MO, USA). Isoelectric focusing and Western blotting reagents were from GE Healthcare and 18% Criterion gels were from Bio-Rad (Hercules, CA).

Cell culture

HUVECs were harvested from umbilical cords obtained with informed consent and the study was approved by Upper South A Regional Ethics Committee (ethics reference CTY/02/12/2009). Cells were isolated by collagenase digestion and grown in supplemented M199 medium as described previously [22,28]. HUVECs were grown to confluence ($\sim 6 \times 10^4$ cells/cm^2) at 37 °C with 5% CO_2, in flasks and plates pre-coated with 0.1% (w/v) gelatin. Cells were grown in 24-well or 6-well plates (or in 10 cm dishes for 2D electrophoresis) and used by the 5th passage.

Human Jurkat T-lymphoma cells were acquired from ATCC (Rockville, MD, USA) and cultured at 37 °C and 5% CO_2 in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Jurkat cells in which CypA had been deleted by gene targeting were a generous gift from Professor Jeremy Luban (Geneva, Switzerland), and were maintained in the same culture conditions as the wild type cells.

Exposure of cells to H_2O_2, HOCl and chloramines

Reagent HOCl was diluted in phosphate-buffered saline (PBS, 140 mM NaCl, 13 mM KCl in 10 mM sodium phosphate buffer, pH 7.4) and standardized by dilution in NaOH, final pH > 10, and measuring A_{292} ($\varepsilon = 350\ M^{-1}\ cm^{-1}$). GlyCl and NH_2Cl were prepared in Hanks' balanced salt solution (HBSS; PBS containing 0.5 mM $MgCl_2$, 1 mM $CaCl_2$ and 5.5 mM glucose) by mixing a 10:1 molar excess of glycine or ammonia respectively with HOCl. Their concentrations were determined by reaction with 5-thio-2-nitrobenzoic acid [29]. The stock H_2O_2 was standardized by measuring A_{240} ($\varepsilon = 43.6\ M^{-1}\ cm^{-1}$).

Cultured HUVECs were washed extensively with PBS to remove any medium containing potential scavengers then solutions of HOCl or chloramines in HBSS were added (1 ml to each well for plate assays or 4 ml for 10 cm dishes). Cells were treated for the indicated times at 37 °C, then methionine was added (final concentration 5 mM) to quench any remaining oxidant. The cells were washed with PBS and harvested in lysis buffer. Control cells were treated with HBSS alone. Treatment with H_2O_2 was similar except that the cells were treated in M199 containing fetal bovine serum.

Control and CypA knockout Jurkat cells were treated with stated concentrations of GlyCl in HBSS for 10 min, then with methionine to quench remaining chloramine.

SDS–PAGE and Western blotting

After treatment with oxidant, cells were lysed in buffer containing NEM to alkylate reduced thiols and prevent further disulfide bond formation or cysteine modification. For Western blotting, proteins were separated on non-reducing, 12% acrylamide gels, transferred to PVDF membranes, probed with the relevant antibody and detected using a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence. Bands were visualized using the ChemiDoc XRS gel documentation system and quantified using Quantity One analysis software (Bio-Rad, Hercules, CA, USA).

Oxidized thiol proteins were monitored using IAF as described previously [30–32]. Briefly, after blocking the reduced thiols, excess NEM was removed using Bio-Gel P-6 DG desalting gel columns (Bio-Rad Laboratories). Oxidized thiols were reduced with 1 mM dithiothreitol (DTT) and any newly-reduced thiols were labeled with 200 μM IAF. For 1D SDS–PAGE, excess IAF was removed by solvent precipitation and resolubilization of proteins was accomplished by addition of gel loading buffer. Separated proteins were detected using a Bio-Rad Molecular Imager™ FX (ex 488 nm/em 530 nm).

For 2DE, proteins were separated from excess IAF by buffer exchange into rehydration buffer (7 M urea, 2 M thiourea, 10 mM DTT, 1% Triton X-100, and 0.2% Biolytes) and applied overnight to immobilized pH gradient strips. Strips were focused for 45 kVh and then separated on Criterion™ Tris–HCl 8–16% gradient gels and scanned as described above. Gel images were analyzed with PDQuest™ version 8.0 (Bio-Rad Laboratories). Images from three independent replicates for each treatment were analyzed and spots that differed in intensity from control gels by at least 2-fold were identified using the replicate group comparison mode along with Gaussian spot quantification. A number of changes identified in this way were manually excluded due to low intensity, inconsistent appearance between replicate gels or their being in a streaky area of the gel. A shortlist of spots that showed consistent changes was selected for mass spectrometric analysis.

For spot excision and identification, a pooled IAF-labeled sample from four extractions was separated on a large format (17 cm) 2D gel. Following fluorescence imaging of the gels, spots were manually excised from unstained gels overlaid on their fluorescent images, and the gels were re-imaged to confirm that the correct spots had been removed. Excised spots were sent to the Centre for Protein Research (University of Otago, Dunedin, New Zealand) where they were subjected to in-gel tryptic digestion using a robotic workstation (DigestPro MSi, Intavis AG, Cologne, Germany). Tryptic peptides were concentrated, redissolved in a solution of 5% (v/v) acetonitrile and 0.2% (v/v) formic acid and analyzed by liquid chromatography/mass spectrometry (LC/MS).