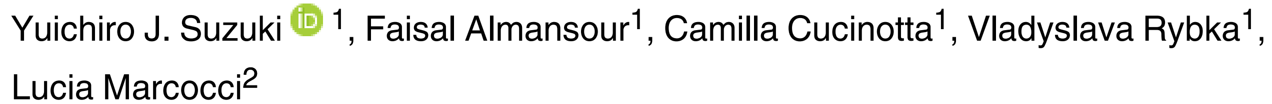
RESEARCH NOTE

Cell signaling promoting protein carbonylation does not cause sulfhydryl oxidation: Implications to the mechanism of redox signaling [version 1; referees: awaiting peer review]



Yuichiro J. Suzuki [1], Faisal Almansour[1], Camilla Cucinotta[1], Vladyslava Rybka[1], Lucia Marcocci[2]

[1]Department of Pharmacology and Physiology, Georgetown University Medical Center, Washington, DC, 20057, USA
[2]Department of Biochemical Sciences "A. Rossi Fanelli", Sapienza University of Rome, Rome, Italy

v1 **First published:** 10 Apr 2017, **6**:455 (doi: 10.12688/f1000research.11296.1)
Latest published: 10 Apr 2017, **6**:455 (doi: 10.12688/f1000research.11296.1)

Abstract

Reactive oxygen species (ROS) have been recognized as second messengers, however, targeting mechanisms for ROS in cell signaling have not been defined. While ROS oxidizing protein cysteine thiols has been the most popular proposed mechanism, our laboratory proposed that ligand/receptor-mediated cell signaling involves protein carbonylation. Peroxiredoxin-6 (Prx6) is one protein that is carbonylated at 10 min after the platelet-derived growth factor (PDGF) stimulation of human pulmonary artery smooth muscle cells. In the present study, the SulfoBiotics Protein Redox State Monitoring Kit Plus (Dojindo Molecular Technologies) was used to test if cysteine residues of Prx6 are oxidized in response to the PDGF stimulation. Human Prx6 has a molecular weight of 25 kDa and contains two cysteine residues. The Dojindo system adds the 15 kDa Protein-SHifter if these cysteine residues are reduced in the cells. Results showed that, in untreated cells, the Prx6 molecule predominantly exhibited the 55 kDa band, indicating that both cysteine residues are reduced in the cells. Treatment of cells with 1 mM H_2O_2 caused the disappearance of the 55 kDa band and the appearance of a 40 kDa band, suggesting that the high concentration of H_2O_2 oxidized one of the two cysteine residues in the Prx6 molecule. By contrast, PDGF stimulation had no effects on the thiol status of the Prx6 molecule. We concluded that protein carbonylation is a more sensitive target of ROS during ligand/receptor-mediated cell signaling than sulfhydryl oxidation.

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Corresponding author: Yuichiro J. Suzuki (ys82@georgetown.edu)

How to cite this article: Suzuki YJ, Almansour F, Cucinotta C *et al.* **Cell signaling promoting protein carbonylation does not cause sulfhydryl oxidation: Implications to the mechanism of redox signaling [version 1; referees: awaiting peer review]** *F1000Research* 2017, **6**:455 (doi: 10.12688/f1000research.11296.1)

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Grant information: This work was supported by the National Institutes of Health, National Heart, Lung, and Blood Institute and National Institute of Aging (Grants R01 HL72844 and R03 AG047824) to YJS. The content is solely the responsibility of the authors and does not represent the official views of the National Institutes of Health.
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: No competing interests were disclosed.

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Introduction

Reactive oxygen species (ROS) have been shown to play important roles in cell signaling (Finkel, 2011; Suzuki *et al.*, 1997). In particular, the roles of ROS in cell growth signaling have been well documented (Rao & Berk, 1992; Sundaresan *et al.*, 1995). For the mechanism of ROS signaling, the receptor activation producing ROS via NAD(P)H oxidase is a widely accepted concept (Griendling *et al.*, 1994). However, molecular targeting mechanisms for ROS in cell signaling have been unclear. ROS targeting protein cysteine thiols has been the most popular proposed mechanism (D'Autreaux & Toledano, 2007; Forman *et al.*, 2010; Moran *et al.*, 2001; Rhee *et al.*, 2000; Sen, 2000; Truong & Carroll, 2012; Veal *et al.*, 2007), yet the occurrence of thiol oxidation requires levels of ROS that are much higher than what is expected to occur during cell signaling (Burgoyne *et al.*, 2007).

Our laboratory has proposed that ligand/receptor-mediated cell signaling involves protein carbonylation (Wong *et al.*, 2008; Wong *et al.*, 2010), which occurs on four susceptible amino acid residues: proline, arginine, lysine, and threonine (Amici *et al.*, 1989; Berlett & Stadtman, 1997). Notably, in cultured cells, hydrogen peroxide (H_2O_2) as low as 0.5 µM was found to promote protein carbonylation (Wong *et al.*, 2008).

More recently, we identified proteins that are carbonylated in response to the platelet-derived growth factor (PDGF) stimulation. Among them, peroxiredoxin-6 (Prx6) was found to be carbonylated in response to a 10-min treatment of human pulmonary artery smooth muscle cells with PDGF (Wong *et al.*, 2013). Peroxiredoxins have been shown to regulate cell signaling (Woo *et al.*, 2010). The present study tested whether this signaling mechanism also promotes sulfhydryl oxidation within the Prx6 molecule.

Methods

HPASMCs (ScienCell Research Laboratories, Carlsbad, CA, USA) were serum-starved overnight and treated with recombinant human PDGF-BB or H_2O_2 for 10, 15 or 30 min. Protein thiol states were monitored using SulfoBiotics Protein Redox State Monitoring Kit Plus (Dojindo Molecular Technologies, Rockville, MD, USA) in accordance with the manufacturer's instructions. Briefly, cells were washed, proteins precipitated with trichloroacetic acid and "Protein-SHifters" were added to each sample. Samples were then loaded onto a sodium dodecyl sulfate polyacrylamide gel and electrophoresed. The gel was exposed to UV light to cut the "Protein-SHifters." The resultant non-reducing SDS polyacrylamide gel was electroblotted to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked with 5% milk for 30 min at room temperature and incubated with the anti-Prx6 antibody produced in rabbit (Sigma-Aldrich Chemical Company, St. Louis, MO, USA; Catalogue no. P0058; 1:1,000 dilution) at 4°C overnight. The membrane was then washed three times and incubated with goat anti-rabbit IgG-horseradish peroxidase conjugate (Bio-Rad; Catalogue no. 1706515; 1:3,000 dilution) for 45 min at room temperature. After washing three times, signals were obtained using an Enhanced Chemiluminescence System (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA).

Results

The technology developed for SulfoBiotics Protein Redox State Monitoring Kit Plus, by Dojindo Molecular Technologies adds a 15 kDa Protein-SHifter on free sulfhydryl groups, allowing the visualization of the thiol status of a given protein by coupling with immunoblotting. The human Prx6 molecule with a molecular weight of 25 kDa has two cysteine residues. Our results indicated that untreated human pulmonary artery smooth muscle cells predominantly contain the 55 kDa species, consistent with the Prx6 molecule, which has two Protein-SHifters incorporated, indicating that both cysteine residues occur in the reduced form in the cells (Figure 1A, lane 1). Treatment of cells with PDGF (10 ng/ml) for 10 min, which promoted protein carbonylation of Prx6 (Wong *et al.*, 2013), did not alter the thiol state of Prx6 (Figure 1A, lane 1 and lane 2). The PDGF treatment

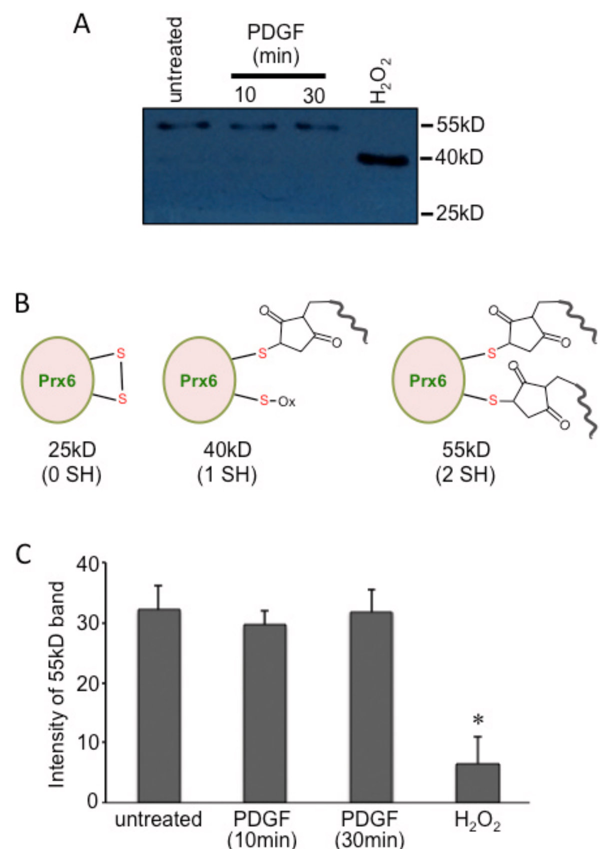

Figure 1. The thiol state of the Prx6 molecule is not altered by PDGF stimulation. Human pulmonary artery smooth muscle cells were treated with PDGF (10 ng/ml) for 10 or 30 min as described in Wong *et al.* (2013), or with H_2O_2 (1 mM) for 15 min. Cellular proteins were precipitated with trichloroacetic acid and lysate samples were prepared in accordance with the manufacturer's instructions for SulfoBiotics Protein Redox State Monitoring Kit Plus (Dojindo). The Protein-SHifter Plus that covalently binds to reduced protein thiols was added and the samples were subjected to electrophoresis through a 12% polyacrylamide gel. Each Protein SHifter Plus causes ~15 kDa shift of the protein bands. After electrophoresis, the gel was exposed to UV irradiation to excise the Protein-SHifter Plus moiety, and then subjected to electrotransfer to a nitrocellulose membrane and Western blotting with the Prx6 antibody. (**A**) Representative Western blotting image of six experiments. (**B**) Diagram of the native 25 kDa Prx6 molecule, the 40 kDa Prx6 molecule with one Protein-SHifter attached, and the 55 kDa Prx6 molecules with two Protein-SHifters attached. (**C**) The bar graph represents means (± SEM) of the intensity of the 55 kDa band (N = 5). The symbol (*) denotes that the value is significantly different from all other values.

for 30 min did not alter the thiol state of Prx6 either (Figure 1A, lane 1 and lane 3). By contrast, treatment of H_2O_2 at a high concentration (1 mM) eliminated the 55 kDa band and generated a 40 kDa band that is consistent with one sulfhydryl group being oxidized (Figure 1A, lane 4). These results were reproduced at least five times. Dataset 1 (Suzuki *et al.*, 2017) contains the uncropped version of Figure 1A and the uncropped repeats. The bar graph shows the data from five separate experiments with five separate cell treatments. Control experiments were performed to ensure that PDGF stimulated protein phosphorylation as well as carbonylation.

Dataset 1. The uncropped version of Figure 1A and the uncropped repeats

http://dx.doi.org/10.5256/f1000research.11296.d157362

Discussion

Unlike protein carbonylation of Prx6, which is promoted in response to PDGF-treatment of human pulmonary artery smooth muscle cells (Wong *et al.*, 2013), PDGF stimulation of cells does not cause the oxidation of two cysteine residues within the human Prx6 molecule. By contrast, cysteine oxidation within the Prx6 molecule can be promoted by treating cells with mM concentrations of H_2O_2 that are not likely to be generated in ligand/receptor-mediated cell signaling. We conclude that protein carbonylation, but not sulfhydryl oxidation, is a likely ROS-targeting mechanism for growth factor stimulation and cell signaling.

Protein carbonylation is promoted by metal-catalyzed generation of hydroxyl radicals, which are known to promote oxidation indiscriminately. However, the caged and site-directed production of hydroxyl radicals via metals could confer specificity (Stadtman & Berlett, 1991; Wong *et al.*, 2010).

Data availability

Dataset 1. The uncropped version of Figure 1A and the uncropped repeats.

DOI, 10.5256/f1000research.11296.d157362 (Suzuki *et al.*, 2017)

Author contributions

YJS conceived the study and designed the experiments. CC, FA, LM, VR, and YJS carried out the research. YJS prepared the first draft of the manuscript. All authors were involved in the revision of the draft manuscript and have agreed to the final content.

Competing interests

No competing interests were disclosed.

Grant information

This work was supported by the National Institutes of Health, National Heart, Lung, and Blood Institute and National Institute of Aging (Grants R01 HL72844 and R03 AG047824) to YJS. The content is solely the responsibility of the authors and does not represent the official views of the National Institutes of Health.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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