RESEARCH NOTE

# Cell signaling promoting protein carbonylation does not cause sulfhydryl oxidation: Implications to the mechanism of redox signaling [version 1; referees: 1 approved, 2 approved with reservations]

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## 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.

**Open Peer Review**

**Referee Status:** ? ✓ ?

| | Invited Referees 1 | 2 | 3 |
|---|---|---|---|
| **version 1** published 10 Apr 2017 | ? report | ✓ report | ? report |

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**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

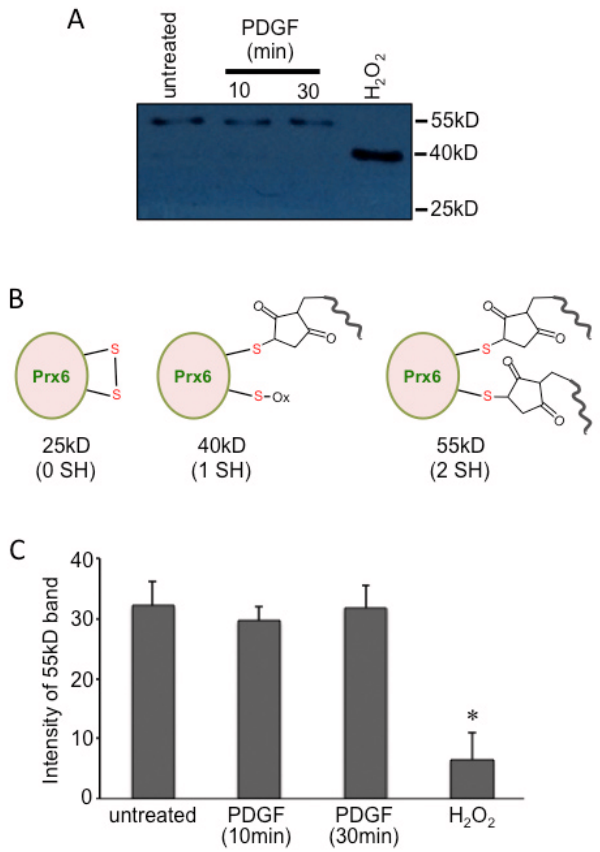


**Figure 1. The thiol state of the Prx6 molecule is not altered by PDGF stimulation.** Human pulmonary artery smooth muscle cells were treated with PDGF (10 ng/ml) for 10 or 30 min as described in Wong *et al.* (2013), or with $H_2O_2$ (1 mM) for 15 min. Cellular proteins were precipitated with trichloroacetic 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**

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## 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

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


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**PubMed Abstract** | **Publisher Full Text**

# Open Peer Review

## Current Referee Status: ? ✔ ?

**Version 1**

Referee Report 15 May 2017

**doi:**10.5256/f1000research.12189.r22633

? **Brian McDonagh**
Department of Physiology, NUI Galway, Galway, Ireland

The authors describe the effects of PDGF and H2O2 treatment on the oxidation state of Prdx6 using a thiol probe, that when attached to free thiols increases the molecular weight of the protein by 15 kDa for each probe attached to the protein. The authors demonstrate that H2O2 treatment causes a change in the redox status of Prdx6 as compared to PDGF treatment. There are a number of issues that need to be resolved and validated by the authors before they can make some of the statements made within the manuscript.

It is essential that the authors fully describe the sample preparation before analysis as this could greatly affect the results and interpretations made. In Figure 1 the authors describe that Prdx6 when the “Protein-SHifter” is added the protein has a mol weight of 55 kDa in controls and the PDGF treatments and one free thiol with the H2O2 treatment, but in Fig1B they show the native state of Prdx6 forming an intra- disulphide, was a reducing agent used in the sample preparation to reduce this disulphide? Does the catalytic Cys47 of this 1-Cys peroxiredoxin form an intra-disulphide with Cys91? It would also be helpful if a non “Protein-SHifter” treated sample was included in the blot to demonstrate the native band at 25 kDa. From Fig1A it would appear that there is a much more intense band for Prdx6 in the H2O2 treated samples, is there a loading control that can be included for this blot?

Carbonylation usually refers to the introduction of an aldehyde or ketone group on an amino acid, I am not sure if this is what the authors are referring to in the title and throughout the manuscript. It is well known that Cys47 of Prdx6 forms a sulphinic (-SO2H) and/or sulphonic (-SO3H) acid. Indeed Prdx6 has been described as having quite a number of various modifications (Jeong, J et al, Proteomics, 2012) so the authors need to confirm the carbonylation or other modifications by mass spectrometry. It is clear that one of the Cys residues is not amenable to “Protein-SHifter” after H2O2 treatment, it would be helpful if they could identify which cysteine residue is susceptible to oxidation.

**Is the work clearly and accurately presented and does it cite the current literature?**
Partly

**Is the study design appropriate and is the work technically sound?**
Partly

**Are sufficient details of methods and analysis provided to allow replication by others?**
No

**If applicable, is the statistical analysis and its interpretation appropriate?**
Yes

**Are all the source data underlying the results available to ensure full reproducibility?**
Partly

**Are the conclusions drawn adequately supported by the results?**
No

***Competing Interests:*** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Referee Report 02 May 2017

**doi:**10.5256/f1000research.12189.r22403

**Tanea T. Reed**
Department of Chemistry, Eastern Kentucky University, Richmond, KY, USA

The authors studied the response of the antioxidant protein, peroxiredoxin-6 to treatment with PDGF and hydrogen peroxide. By using a commercially available kit, the authors discovered oxidation in one of the cysteine residues at high concentrations of H2O2. My only issue with this work is for Figure 1A. The authors state that they tested three time points of hydrogen peroxide, but only one is shown in the figure. By showing all three time points could further verify the finding of this report as the 40kD would be most potentially pronounced at 30 min.

**Is the work clearly and accurately presented and does it cite the current literature?**
Yes

**Is the study design appropriate and is the work technically sound?**
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Yes

**Are all the source data underlying the results available to ensure full reproducibility?**
Partly

**Are the conclusions drawn adequately supported by the results?**
Yes

***Competing Interests:*** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Referee Report 25 April 2017

**doi:**10.5256/f1000research.12189.r22184

**Sabah N.A. Hussain**
Meakins-Christie Laboratories, Translational Research in Respiratory Diseases Program, Department of Critical Care Medicine, Research Institute of the MUHC, McGill University Health Centre (MUHC), Montreal, QC, Canada

The authors provided indirect evidence that peroxiredoxin-6 does not undergo sulfhydryl oxidation when human pulmonary artery smooth muscle cells are exposed to PDGF but this protein undergo sulfhydryl oxidation when these cells were exposed to $H_2O_2$. It was concluded that protein carbonylation is more sensitive target of ROS during ligand/receptor-mediated cell signaling than sulfhydrul oxidation.

Major comments: I believe that the conclusion of this study is too general and the authors should restrict themselves to the main findings of this study and do not extend their observation beyond one type of cells exposed to one growth factor (PDGF).

In addition, the authors used an indirect method to assess sulfydryl oxidation rather than a direct measurement. Moreover, the authors did not provide evidence in the current study that PDGF actually produced carbonylation of Prx6. This data is required to document the differential oxidation response of this protein to these two interventions (H2O2 vs. PDGF exposure).

Finally, the authors need to provide data as to the time course of Prx6 oxidation in response to H2O2 exposure. They have only shown one time point.

**Is the work clearly and accurately presented and does it cite the current literature?**
Yes

**Is the study design appropriate and is the work technically sound?**
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Yes

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
No

***Competing Interests:*** No competing interests were disclosed.

***Referee Expertise:*** Angiogenesis, ROS signaling, NO biology

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**