Published in final edited form as:

Free Radic Biol Med. 2013 December ; 65: . doi:10.1016/j.freeradbiomed.2013.09.005.

Mechanism of protein decarbonylation

Chi-Ming Wong^{1,§}, Lucia Marcocci^{2,§}, Dividutta Das¹, Xinhong Wang^{1,3}, Haibei Luo¹, Makhosazane Zungu-Edmondson¹, and Yuichiro J. Suzuki¹

¹Department of Pharmacology and Physiology, Georgetown University Medical Center, Washington DC 20057 USA

²Department of Biochemical Sciences "A. Rossi Fanelli", Sapienza University of Rome, Rome, Italy

³Department of Physiology and Pathophysiology, Fudan University Shanghai Medical College, Shanghai 200032, China

Abstract

Ligand/receptor-stimulation of cells promotes protein carbonylation that is followed by the decarbonylation process, which might involve thiol-dependent reduction (Wong et al., *Circ. Res.* 102 301-318, 2008). The present study further investigated the properties of this protein decarbonylation mechanism. We found that the thiol-mediated reduction of protein carbonyls is dependent on heat-labile biologic components. Cysteine and glutathione were found to be efficient substrates for decarbonylation. Thiols decreased the protein carbonyl content, as detected by 2,4-dinitrophenylhydrazine, but not the levels of malondialdehyde or 4-hydroxynonenal protein adducts. Mass spectrometry identified proteins that undergo thiol-dependent decarbonylation, which include peroxiredoxins. Peroxiredoxins-2 and -6 were found to be carbonylated and subsequent decarbonylated in response to the ligand/receptor-stimulation of cells. siRNA knockdown of glutaredoxin inhibited the decarbonylation of peroxiredoxin. These results strengthen the concept that thiol-dependent decarbonylation defines the kinetics of protein carbonylation signaling.

Keywords

Carbonylation; Decarbonylation; Oxidant signaling; Oxidative stress; Peroxiredoxin; Protein oxidation; Reactive oxygen species; Redox signaling

Introduction

Reactive oxygen species (ROS)¹ play important roles in the pathogenesis of various diseases, such as cancer, cardiovascular diseases and neurological disorders, as well as in the aging process through their abilities to oxidize and damage biological molecules [1-6]. ROS are also thought to regulate cell signal transduction [7-13], yet the mechanism of how ROS

© 2013 Elsevier Inc. All rights reserved.

To whom correspondence should be addressed: Yuichiro J. Suzuki, Ph.D., Department of Pharmacology and Physiology, Georgetown University Medical Center, 3900 Reservoir Road NW, Washington, DC 20057 USA, TEL: (202) 687-8090, FAX: (202) 687-8825, ys82@georgetown.edu.

[§]These authors contributed equally to this project.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

react with target molecules to serve as second messengers is not well understood. ROS can oxidize a variety of molecules including proteins, lipids, and DNA [1-3]. Protein oxidation through carbonylation has been shown to occur in various diseases and in aging [3,14-19]. We have also reported that protein carbonylation plays a role in the mechanism of ligand/receptor-mediated ROS signaling [20,21].

Our observations for the role of protein carbonylation in cell signaling was also accompanied by the finding that the formed protein carbonyls can be decreased in the process, which we termed “protein decarbonylation” [20]. We found that the promotion of protein carbonylation as detected using 2,4-dinitrophenylhydrazine (DNPH)¹ [22] is followed by decarbonylation in response to cell stimulation with ligands [20]. Since secondary protein carbonyls (i.e. addition of aldehydes to amino acid side chains) did not exhibit transient kinetics in response to the ligand stimulation and since protein carbonylation and decarbonylation kinetics were also observed using biotin hydrazide, the decarbonylation process may involve the reduction of primary protein carbonyls [23], in which carbonyls are formed through direct oxidation of amino acid side chains [3,16]. Further, the *in vitro* addition of a thiol reductant, β -mercaptoethanol (BME)¹, decreased protein carbonyl content in smooth muscle cell (SMC)¹ lysates [20]. This was surprising because protein carbonyls are generally thought not to be reduced, particularly by thiols.

The present study further examined these events, in which protein carbonyls are decreased by thiols. Here, we present evidence for the existence of heat-labile cellular components that can catalyze the reduction of protein carbonyls.

Materials and Methods

Experimental animals

Male Sprague-Dawley rats (~200 g) were purchased from Charles River Laboratories (Wilmington, MA, USA) and fed normal rat chow. Tissues were homogenized using a Polytron (Kinematica AG, Switzerland) in four volumes of ice-cold buffer [24]. The Georgetown University Animal Care and Use Committee approved all animal experiments, and the investigation conforms to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cell culture

Human pulmonary artery SMCs (from ScienCell Research Laboratories, Carlsbad, CA, USA) were cultured in accordance with the manufacturer's instructions in 5% CO₂ at 37°C. Cells were serum-starved overnight and treated with human recombinant platelet-derived growth factor (PDGF)¹-BB (Invitrogen, Carlsbad, CA, USA). For siRNA knockdown, cells were transfected with siRNA Transfection Reagent and gene silencing siRNAs for thioredoxin (Trx)¹ and glutaredoxin-1 (Grx1)¹ along with a control siRNA with scrambled sequences (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for two days. Cell lysates were prepared as described previously [20].

Immunoblotting

Carbonyl groups in protein side chains were derivatized with DNPH [22] to form the 2,4-dinitrophenyl (DNP)¹ hydrazone derivative for detection using the Oxyblot Protein

¹Abbreviations 2-D two-dimensional, a.u. arbitrary unit, BME β -mercaptoethanol, BSA bovine serum albumin, DNCB 2,4-dinitrochlorobenzene, DNP 2,4-dinitrophenyl, DNPH 2,4-dinitrophenylhydrazine, DTT dithiothreitol, Cys L-cysteine, ECL enhanced chemiluminescence, IEF isoelectric focusing, Grx1 glutaredoxin-1, GSH L-glutathione, HRP horseradish peroxidase, NAC N-acetyl-L-cysteine, PDGF platelet-derived growth factor, Prx peroxiredoxin, ROS reactive oxygen species, SDS sodium dodecylsulfate, SMC smooth muscle cells, Trx thioredoxin

Oxidation Detection Kit (Millipore, Billerica, MA, USA), according to the manufacturer's instructions. To study the effects of thiols on carbonyl content, some tissue homogenates/cell lysates (20 µg protein) or purified bovine serum albumin (BSA¹; Sigma-Aldrich, St. Louise, MO, USA; 1.5 µg) were treated with BME (Bio-Rad Laboratories, Hercules, CA, USA) or other reductants including L-cysteine (Cys)¹, N-acetyl-L-cysteine (NAC)¹, L-glutathione (GSH)¹, and dithiothreitol (DTT)¹ (Sigma-Aldrich) for 15 min at room temperature before the addition of sodium dodecylsulfate (SDS)¹ and DNPH. In some experiments, 2,4-dinitrochlorobenzene (DNCB)¹ (Sigma-Aldrich) was added before the addition of reductants. After neutralization, BME was again added at final 2% to all the samples for performing reducing 12% SDS-polyacrylamide gel electrophoresis and electroblotting onto a nitrocellulose membrane (Bio-Rad). The membrane was then blocked with 1% BSA (Sigma-Aldrich; Cat# A7638) for 1 h at room temperature and incubated overnight at 4°C with rabbit anti-DNP antibody (OxyBlot; 1:150 dilution). The levels of carbonylated proteins were detected with horseradish peroxidase (HRP)¹-conjugated goat anti-rabbit IgG (OxyBlot; 1:300 dilution) and the enhanced chemiluminescence (ECL)¹ system (Amersham Biosciences, Piscataway, NJ, USA). The developed films were scanned with an imaging densitometer, and optical densities of protein bands were quantified using the NIH ImageJ version 1.43.

Other modifications of proteins were detected by treating heart homogenates with or without BME or Cys for 15 min at room temperature. Samples were then subjected to reducing 12% SDS-polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane. The membrane was then blocked with 5% milk (Bio-Rad) for 1 h at room temperature and incubated overnight at 4°C with antibodies against malondialdehyde (MDA; Alpha Diagnostic International, San Antonio, TX; Cat# MDA11-S; rabbit polyclonal; 1:5,000 dilution), or 4-hydroxynonenal (4-HNE; Alpha Diagnostic; Cat# HNE11-S; rabbit polyclonal; 1:2,000 dilution), followed by incubations with the HRP-conjugated secondary antibody (Bio-Rad; 1:3,000 dilution) and the ECL detection.

For immunoprecipitation experiments, 4 volumes (160 µl) of 10 mM DNPH (in 2 M HCl) were added to cell lysates (50 µg protein in 40 µl). Ice-cold trichloroacetic acid (15% w/v final) was added, incubated for 10 min on ice, and centrifuged for 10 min. The pellet was washed with 400 µl ethanol ethyl acetate (1:1) twice, resuspended in 100 µl of the lysis buffer, and incubated with 1 µl anti-DNP IgG and 10 µl GammaBind G-Sepharose (Amersham) overnight at 4°C with gentle shaking. After washing twice, the pellet was boiled in Laemmli buffer and centrifuged, and the supernatant was subjected to reducing 12% SDS-polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane. The membrane was then blocked with 5% milk for 1 h at room temperature and incubated overnight at 4°C with antibodies against peroxiredoxin (Prx)¹-2 (Santa Cruz; Cat# sc-23967; goat polyclonal; 1:500 dilution) and Prx-6 (Santa Cruz; Cat# sc-59671; mouse monoclonal; 1:500 dilution), followed by incubations with HRP-conjugated secondary antibodies (Bio-Rad and Santa Cruz; 1:2,000 dilution) and the ECL detection.

The effectiveness of knocking down Trx and Grx1 by siRNA was also assessed by immunoblotting using antibodies against Trx (Santa Cruz; Cat# sc-18215; goat polyclonal; 1:200 dilution) and Grx1 (Santa Cruz; Cat# sc-32943; rabbit polyclonal; 1:500 dilution), followed by incubations with HRP-conjugated secondary antibodies (Bio-Rad; 1:2,000 dilution) and the ECL detection.

Two-dimensional gel electrophoresis and protein identification by mass spectrometry

Carbonylated proteins were identified as previously described [20]. Briefly, four volumes of 10 mM DNPH were added to protein samples (200 µg) and incubated for 30 min, followed by incubation with 15% trichloroacetic acid for 10 min. Samples were then centrifuged for

10 min, the pellet was washed three times with ethanol ethyl acetate, and samples were centrifuged for 15 min. The pellet was resuspended in two-dimensional (2-D)¹ rehydration buffer (Bio-Rad). The first-dimension of protein separation was performed using the Protean Isoelectric Focusing (IEF)¹ Cell (Bio-Rad) with pH gradient strips (nonlinear pH 5 - 8). For the second dimension, IEF strips were embedded in agarose on top of 10.5 - 14% acrylamide gels for SDS-PAGE, followed by electrotransfer to nitrocellulose membranes and immunoblotting with the rabbit anti-DNP IgG antibody (OxyBlot; 1:150 dilution) and HRP-conjugated goat anti-rabbit IgG (OxyBlot; 1:300 dilution).

Carbonylated protein spots of interest were excised from Coomassie blue stained gels. Mass spectra were recorded with a matrix assisted laser desorption/ionization–time of flight, time of flight (MALDI-TOF-TOF) spectrometer (4800 Proteomics Analyzer, Framingham, MA, USA). Samples were ionized with a fixed laser intensity of 3,500 J, and 1,000 laser shots were collected per spectrum. The detector voltage was 2.1 kvolts, the bin size was set at 0.5 nsec and the signal/noise threshold was set at 10. The spectra were collected with a specified mass range of 799 – 4,000 daltons with a focus mass of 2,000 daltons. Peptide masses were compared with the theoretical masses derived from the sequences contained in SWISS-PROT/NCBI databases using MASCOT.

Statistical analysis

Comparisons between two groups were analyzed by a two-tailed Student's *t* test, and comparisons between three or more groups were analyzed by ANOVA with a Student-Newman-Keuls post-hoc test. *P* < 0.05 was considered to be significant.

Results

We have previously shown that the addition of 2% (w/v) BME before the incubation with DNPH to lysates prepared from cultured cells decreases the protein carbonyl content [20]. In the present study, we first tested to see if this event also occurs in tissues. Tissue homogenates of rat heart, kidney, liver and brain were treated with or without BME. DNPH was then added and reducing SDS-PAGE and immunoblotting were used to detect protein carbonyls. While all these tissues exhibited multiple carbonylated proteins, carbonyl signals were consistently decreased by the addition of BME only in the rat heart homogenates (Fig. 1A). BME also decreased the carbonyl content in mouse heart homogenates (Supplemental Figure S1). BME-mediated decrease in the carbonyl content was observed in both left and right heart ventricles (data not shown).

To determine if protein carbonyls can directly be reduced by BME, purified BSA protein was treated with BME before derivatization with DNPH. We found that BME did not influence the carbonyl content of purified BSA (Fig. 1B). These results indicate that protein carbonyls are not chemically reduced by thiols and suggest that the heart may possess cellular components that catalyze thiol-dependent reduction of protein carbonyls.

To test this hypothesis, the heart homogenates were heated at 60°C to inactivate cellular protein activities before the addition of BME. We found that heating inhibited BME-dependent reduction of protein carbonylation (Fig. 2).

We previously proposed that the decarbonylation mechanism, which occurs in response to the treatment of cells with a ligand, is mediated by the Trx/Trx reductase system [20]. Thus, we tested if Trx reductase is involved in the BME-induced reduction of protein carbonyls *in vitro*. The addition of DNCB, a Trx reductase inhibitor, however, did not influence the BME-mediated reduction of protein carbonyls (Fig. 3). Thus, Trx reductase does not appear

to be the heat-labile enzyme that can catalyze the BME-induced decrease in the carbonyl content.

We next examined which reductants, in addition to BME, may decrease the protein carbonyl contents. We found that various thiol reductants including Cys, NAC, GSH, and DTT at 2% (w/v) also decreased the carbonyl content in the heart tissue homogenates (data not shown). Among them, Cys and GSH exhibited the strongest ability to decrease the protein carbonyl content. As shown in Fig. 4, Cys and GSH significantly decreased the carbonyl content at concentrations as low as 250 μ M, i.e. 0.003% (w/v) for Cys and 0.008% (w/v) for GSH.

Protein carbonyls as detected by DNPH can be either primary or secondary carbonyls [16]. We found that protein adducts of aldehydes, such as MDA and 4-HNE, which form secondary protein carbonyls, were not influenced by thiols. While BME and Cys dose-dependently decreased the DNPH signals in the rat heart homogenates, neither of these thiols decreased MDA protein adduct levels even at the highest concentration used (Fig. 5). Similarly, BME did not decrease the 4-HNE adduct signals (Supplemental Figure S2).

To identify proteins whose carbonyl contents can be decreased by thiols, 2-D gel electrophoresis was performed and carbonylated protein spots were visualized by immunoblotting. We identified four carbonylated spots that consistently showed BME-induced reduction. Supplemental Figure S3A shows the location of the four-carbonylated proteins in the 2-D immunoblots with or without BME (2%) treatment before the DNPH derivatization. Supplemental Figure S3B shows the corresponding Coomassie blue stained gel. Densitometry analysis determined that BME significantly reduced the carbonyl content of these proteins relative to total protein expression (Supplemental Figure S4). Mass spectrometry identified these carbonylated proteins as listed in Table 1.

We found that, among four proteins identified to undergo thiol-mediated decarbonylation, three of them were Prx antioxidant enzymes. Immunoprecipitation and immunoblotting confirmed that both BME and Cys at 2% (w/v) decreased the carbonyl contents of Prx-2 and -6 (Supplemental Figure S5). Cys exhibited a strong activity to decrease the carbonyl content of Prx-2 and -6 with 0.003% (w/v) being sufficient to cause a significant reduction (Fig. 6A).

We previously showed that the ligand/receptor activation of cultured pulmonary artery SMCs resulted in the carbonylation of various proteins (with a peak at 10 min) and subsequent decarbonylation (which occurs at 30 min) [20]. Thus, we tested if carbonylation and decarbonylation of Prxs may occur in cultured pulmonary artery SMCs. Our results demonstrate that the treatment of human pulmonary artery SMCs with PDGF promoted the carbonylation of Prx-2 and -6 at 10 min, followed by decarbonylation at 30 min after stimulation (Fig. 6B).

To test if Trx may regulate the decarbonylation phase of ligand-mediated Prx carbonylation, Trx was knocked down in human pulmonary artery SMCs by siRNA (Supplemental Figure S6A). However, knocking down Trx did not influence the PDGF-mediated decarbonylation of Prx6 (Fig. 7A). By contrast, siRNA knockdown of Grx1 (Supplemental Figure S6B) completely suppressed the decarbonylation mechanism for Prx6 in PDGF-treated cells (Fig. 7B).

To determine the effects of knocking-down Trx and Grx1 in the thiol-dependent decrease in protein carbonyl content *in vitro*, cell lysates from human pulmonary SMCs were treated with BME after the siRNA knockdown. Results show that BME decreases the Prx6 carbonyl content in the lysates from Trx knocked-down cells (Fig. 8A). By contrast, knocking-down Grx1 completely inhibited the decrease in Prx6 carbonyl content induced by BME (Fig. 8B).

Discussion

We previously reported that the treatment of cultured pulmonary artery SMCs with endothelin-1 promoted the receptor-dependent protein carbonylation that was followed by the reduction of formed carbonyls, which we named “protein decarbonylation” [20]. We also proposed that this transient kinetics of cell signaling-mediated protein carbonylation is due to thiol-dependent reduction of the protein carbonyl content during the decarbonylation phase [20,21]. One experimental finding that supported this concept was the surprising observation that the addition of BME to the cell lysate samples before the addition of DNPH decreased protein carbonyl content [20]. The present study extends these previous findings by demonstrating that the thiol-dependent decrease in protein carbonyls also occurs in the rat and mouse heart tissue homogenates and providing evidence for the existence of heat-labile biologic components, which may catalyze the thiol-dependent decarbonylation.

It is not generally thought that thiols can reduce protein carbonyls. Indeed, our experiments indicated that BME is not capable of decreasing the carbonyl content in a purified protein. From these results, we hypothesize that biological components in pulmonary vascular SMC extracts and in heart tissue homogenates contain catalysts for thiol-dependent protein decarbonylation. The experiments presented in this study support this hypothesis by showing that heating the heart tissue homogenates to 60°C to inactivate protein functions inhibits the BME-induced decrease in protein carbonyl content. Other thiols, notably Cys and GSH, are also effective in decreasing protein carbonyls.

DNPH that was used in this study can detect both primary and secondary protein carbonyls [25]. Primary protein carbonylation refers to the formation of carbonyls through direct oxidation of amino acid side chains, while secondary protein carbonylation refers to the introduction of carbonyl-containing molecules, such as aldehydes, to amino acid side chains [14,16,26]. Results from the present study demonstrate that neither 4-HNE adducts nor MDA adducts are decreased by thiols. Thus, thiol-sensitive components that can be labeled by DNPH may not be secondary carbonyls, although other types of secondary carbonyls cannot be excluded from our studies. As DNPH is a reliable method to detect protein carbonyls and has been used in a number of investigations [3,14,15,17-19,22,25-27], it is likely that the majority of DNPH signals that can be eliminated by reductants are protein carbonyls, rather than other possible chemical reactions such as sulfenic acid [23,28].

Among various carbonylated proteins that can be affected by the thiol reduction, we identified four proteins in the rat heart homogenates that undergo thiol-induced decarbonylation using mass spectrometry. It is interesting to note that three out of the four proteins that we identified to be decarbonylated are Prxs. Immunoprecipitation experiments confirmed that the carbonyl contents of Prxs are indeed decreased by thiols. Further, in cultured pulmonary artery SMCs, we found that Prxs undergo carbonylation and decarbonylation in response to the ligand stimulation at 10 and 30 min, respectively. Thus, the reduction of carbonyls in Prx molecules may play a role in regulating cell signaling. This may be related to a regulatory mechanism of redox signaling proposed by Rhee and co-workers [9], in which Prxs are transiently inactivated in response to the receptor stimulation for fine-tuning ROS signaling.

We previously proposed that Trx may act as a biologic thiol, which serves as an electron donor, because ligand/receptor-mediated decarbonylation was inhibited by DNCB, a Trx reductase inhibitor [20]. Our experiments using DNCB, however, suggest that Trx reductase does not directly play a role in the BME-mediated decarbonylation process. Further, siRNA knockdown of Trx did not inhibit the occurrence of Prx-6 decarbonylation in pulmonary artery SMCs. One explanation for these observations is that the knockdown of Trx may have

upregulated other thiols as an adaptation. Alternatively, DNCB may also inhibit other enzymes and the Trx/Trx reductase system may not regulate the decarbonylation mechanism. Interestingly, the present study provides evidence for the role of Grx1, which is known to promote deglutathionylation [29], in regulating protein decarbonylation.

In summary, the present study provides evidence for the existence of a heat-labile mechanism, which supports the thiol-dependent decrease in protein carbonyl content. We propose that the biological system contains the enzymatic system that catalyzes the elimination of formed protein carbonyls in the process of protein decarbonylation. Decarbonylated proteins include Prxs, which may regulate ROS signaling. Our data revealed that Grx1 is involved in the process of Prx6 decarbonylation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by grants from the National Institutes of Health (R01HL72844 and R01HL97514) and the American Heart Association Mid-Atlantic Affiliate to YJS.

References

- Halliwell, B.; Gutteridge, J., editors. *Free Radicals in Biology and Medicine*. Oxford: Oxford University Press; 2007.
- Freeman BA, Crapo JD. Biology of disease: free radicals and tissue injury. *Lab Invest*. 1982; 47:412–426. [PubMed: 6290784]
- Berlett BS, Stadtman ER. Protein oxidation in aging, disease, and oxidative stress. *J Biol Chem*. 1997; 272:20313–20316. [PubMed: 9252331]
- Zhang Y, Tocchetti CG, Krieg T, Moens AL. Oxidative and nitrosative stress in the maintenance of myocardial function. *Free Radic Biol Med*. 2012; 53:1531–1540. [PubMed: 22819981]
- Labunskyy VM, Gladyshev VN. Role of reactive oxygen species-mediated signaling in aging. *Antioxid Redox Signal*. 2012 In press.
- Halliwell B. Oxidative stress and cancer: have we moved forward? *Biochem J*. 2007; 401:1–11. [PubMed: 17150040]
- Suzuki YJ, Forman HJ, Sevanian A. Oxidants as stimulators of signal transduction. *Free Radic Biol Med*. 1997; 22:269–285. [PubMed: 8958153]
- Rhee SG, Bae YS, Lee SR, Kwon J. Hydrogen peroxide: a key messenger that modulates protein phosphorylation through cysteine oxidation. *Sci STKE*. 2000; 2000:pe1. [PubMed: 11752613]
- Woo HA, Yim SH, Shin DH, Kang D, Yu DY, Rhee SG. Inactivation of peroxiredoxin I by phosphorylation allows localized H₂O₂ accumulation for cell signaling. *Cell*. 2010; 140:517–528. [PubMed: 20178744]
- Truong TH, Carroll KS. Redox regulation of epidermal growth factor receptor signaling through cysteine oxidation. *Biochemistry*. 2012; 51:9954–9965. [PubMed: 23186290]
- Forman HJ, Maiorino M, Ursini F. Signaling functions of reactive oxygen species. *Biochemistry*. 2010; 49:835–842. [PubMed: 20050630]
- Burgoyne JR, Madhani M, Cuello F, Charles RL, Brennan JP, Schröder E, Browning DD, Eaton P. Cysteine redox sensor in PKGI α enables oxidant-induced activation. *Science*. 2007; 317:1393–1397. [PubMed: 17717153]
- Finkel T. Signal transduction by reactive oxygen species. *J Cell Biol*. 2011; 194:7–15. [PubMed: 21746850]
- Levine RL. Carbonyl modified proteins in cellular regulation, aging, and disease. *Free Radic Biol Med*. 2002; 32:790–796. [PubMed: 11978480]

15. Dalle-Donne I, Aldini G, Carini M, Colombo R, Rossi R, Milzani A. Protein carbonylation, cellular dysfunction, and disease progression. *J Cell Mol Med*. 2006; 10:389–406. [PubMed: 16796807]
16. Suzuki YJ, Carini M, Butterfield DA. Protein carbonylation. *Antioxid Redox Signal*. 2010; 12:323–325. [PubMed: 19743917]
17. Butterfield DA, Perluigi M, Reed T, Muharib T, Hughes CP, Robinson RA, Sultana R. Redox proteomics in selected neurodegenerative disorders: from its infancy to future applications. *Antioxid Redox Signal*. 2012; 17:1610–1655. [PubMed: 22115501]
18. Barreiro E, Hussain SN. Protein carbonylation in skeletal muscles: impact on function. *Antioxid Redox Signal*. 2010; 12:417–429. [PubMed: 19686036]
19. Nyström T. Role of oxidative carbonylation in protein quality control and senescence. *EMBO J*. 2005; 24:1311–1317. [PubMed: 15775985]
20. Wong CM, Cheema AK, Zhang L, Suzuki YJ. Protein carbonylation as a novel mechanism in redox signaling. *Circ Res*. 2008; 102:310–318. [PubMed: 18079412]
21. Wong CM, Marcocci L, Liu L, Suzuki YJ. Cell signaling by protein carbonylation and decarbonylation. *Antioxid Redox Signal*. 2010; 12:393–404. [PubMed: 19686045]
22. Levine RL, Williams JA, Stadtman ER, Shacter E. Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol*. 1994; 233:346–57. [PubMed: 8015469]
23. Wong CM, Bansal G, Marcocci L, Suzuki YJ. Proposed role of primary protein carbonylation in cell signaling. *Redox Report*. 2012; 17:90–94. [PubMed: 22564352]
24. Park AM, Nagase H, Vinod Kumar S, Suzuki YJ. Acute intermittent hypoxia activates myocardial cell survival signaling. *Am J Physiol Heart Circ Physiol*. 2007; 292:H751–H757. [PubMed: 17098826]
25. Yan LJ, Forster MJ. Chemical probes for analysis of carbonylated proteins: A review. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2011; 879:1308–1315.
26. Grimsrud PA, Xie H, Griffin TJ, Bernlohr DA. Oxidative stress and covalent modification of protein with bioactive aldehydes. *J Biol Chem*. 2008; 283:21837–21841. [PubMed: 18445586]
27. Madian AG, Regnier FE. Proteomic identification of carbonylated proteins and their oxidation sites. *J Proteome Res*. 2010; 9:3766–3780. [PubMed: 20521848]
28. Dalle-Donne I, Carini M, Orioli M, Vistoli G, Regazzoni L, Colombo G, Rossi R, Milzani A, Aldini G. Protein carbonylation: 2,4-dinitrophenylhydrazine reacts with both aldehydes/ketones and sulfenic acids. *Free Radic Biol Med*. 2009; 46:1411–1419. [PubMed: 19268703]
29. Dalle-Donne I, Rossi R, Giustarini D, Colombo R, Milzani A. S-glutathionylation in protein redox regulation. *Free Radic Biol Med*. 2007; 43:883–898. [PubMed: 17697933]

Highlights

- Thiol reductants decrease the protein carbonyl content in tissue homogenates, but not of a purified protein.
- The thiol-dependent decrease in carbonyl content that occurs in the tissue homogenates is inhibited by heat inactivation.
- Proteins that can be decarbonylated by thiol reductants include peroxiredoxin-2 and -6.
- Ligand stimulation of cells promotes carbonylation and subsequent decarbonylation of peroxiredoxin-2 and -6.
- Glutaredoxin is involved in the decarbonylation mechanism.

Fig. 1. Effects of thiol reduction on protein carbonylation in heart tissue homogenates and purified BSA

(A) Rat heart homogenates were treated with or without 2% (w/v) BME, then derivatized with DNPH to monitor carbonylated protein content by immunoblotting. The bar graph represents means $\pm$ SEM ($n = 10$) of percent of carbonyl content relative to untreated control without the BME treatment. The symbol * denotes that the value is significantly different from the control at $P < 0.05$. Coomassie stained proteins are also shown to demonstrate that BME did not influence total protein levels. (B) BSA was treated with or without 2% (w/v) BME, then derivatized with DNPH to monitor carbonylated protein content by immunoblotting. The bar graph represents means $\pm$ SEM ($n = 8$) of percent of carbonyl content relative to untreated control without BME treatment. The symbol ns denotes that the two values are not significantly different.

Fig. 2. Effects of heating on thiol-mediated reduction on protein carbonylation in heart tissue homogenates

Rat heart homogenates were subjected to heating at 60°C or maintained at room temperature for 30 min. Samples were then treated with or without 2% (w/v) BME, then derivatized with DNPH to monitor carbonylated protein contents by immunoblotting. Coomassie stained proteins are also shown to demonstrate that BME or heating did not influence total protein levels. The bar graph represents means ± SEM (n = 10) of percent of carbonyl content relative to untreated control without BME treatment. The symbol * denotes that the values are significantly different from each other at P < 0.05.

Fig. 3. Effects of a Trx reductase inhibitor on thiol-mediated reduction of protein carbonylation in heart tissue homogenates

Rat heart homogenates were pre-treated with or without 30 μ M DNCB or its vehicle DMSO. Samples were then treated with or without 2% (w/v) BME, then derivatized with DNPH to monitor carbonylated protein contents by immunoblotting. Coomassie stained proteins are also shown to demonstrate that BME did not influence total protein levels. The bar graph represents means $\pm$ SEM ($n = 4$) of percent of carbonyl content relative to untreated control without BME treatment. The symbol * denotes that the values are significantly different from each other at $P < 0.05$.

Fig. 4. Effects of various thiols on protein carbonylation in heart tissue homogenates
 Rat heart homogenates were treated with 250 μ M BME, Cys, NAC, GSH, or DTT, then derivatized with DNPH to monitor carbonylated protein contents by immunoblotting. Coomassie stained proteins are also shown to demonstrate that thiols did not influence total protein levels. The bar graph represents means $\pm$ SEM ($n = 3$) of percent of carbonyl content relative to untreated control without treatment. The symbol * denotes that the value is significantly different from the control at $P < 0.05$.

Fig. 5. Effects of thiol reduction on secondary protein carbonylation in heart tissue homogenates
 Rat heart homogenates were treated with various amounts of (A) BME or (B) Cys, then subjected to immunoblotting to detect protein carbonyls using DNPH and MDA protein adducts. Coomassie stained proteins are also shown to demonstrate that BME or Cys did not influence total protein levels. Line graphs represent means $\pm$ SEM ($n = 3$). The symbol * denote that the value is significantly different from the untreated control at $P < 0.05$.

Fig. 6. Decarboxylation of Prxs

(A) Rat heart homogenates were treated with 250 μ M BME (0.002 w/v %) or Cys (0.003 w/v %) then derivatized with DNPH, immunoprecipitated with the DNP antibody, and immunoblotted with Prx2 or Prx6 antibody. Bar graphs represent means $\pm$ SEM (n = 3). The symbol * denotes that the value is significantly different from the control at P < 0.05. (B) Cultured human pulmonary artery SMCs were treated with PDGF for 10 or 30 min. Cell lysates were prepared, derivatized with DNPH, immunoprecipitated with the DNP antibody, and then immunoblotted with Prx2 or Prx6 antibody. Bar graphs represent means $\pm$ SEM (n = 3). The symbol * denotes that the values are significantly different from each other at P < 0.05.

Fig. 7. Effects of siRNA knockdown of Trx and Grx1 on PDGF-induced carbonylation and decarbonylation in cultured cells

Cultured human pulmonary artery SMCs were transfected with (A) Trx or (B) Grx1 siRNA for 2 days, followed by PDGF stimulation for 10 or 30 min. Cell lysates were prepared, derivatized with DNPH, immunoprecipitated with the DNP antibody, and then immunoblotted with the Prx6 antibody. Bar graphs represent means $\pm$ SEM of the ratio of carbonylated Prx6 to Prx6 protein level expressed in arbitrary unit (a.u.) ($n = 6$ for A; $n = 4$ for B). The symbol * denotes that the values are significantly different from each other at $P < 0.05$. The symbol ns denotes that the two values are not significantly different.

Fig. 8. Effects of siRNA knockdown of Trx and Grx1 on thiol-mediated reduction of protein carbonylation in pulmonary artery SMC lysates

Cultured human pulmonary artery SMCs were transfected with (A) Trx or (B) Grx1 siRNA for 2 days, and cell lysates were prepared. Cell lysates were then treated with or without 2% (w/v) BME, derivatized with DNPH, immunoprecipitated with the DNP antibody, and then immunoblotted with the Prx6 antibody. Bar graphs represent means $\pm$ SEM ($n = 3$ for A; $n = 4$ for B). The symbol * denotes that the values are significantly different from each other at $P < 0.05$. The symbol ns denotes that the two values are not significantly different.

Table 1

List of proteins that can be decarboxylated.

CPS	Protein Name	Accession #	MW	Protein Score	C.I. %
a	Peroxioredoxin-2	P35704	21770	73.5	99.97
b	L-lactate dehydrogenase B chain	P42123	36589	93.1	100
c	Peroxioredoxin-6	O35244	24803	187	100
d	Peroxioredoxin-3	Q9Z0V6	28277	47	85.11

Rat heart homogenates were treated with or without 2% (w/v) BME, then derivatized with DNPH to monitor protein carbonylation using 2-D gel electrophoresis and immunoblotting. Proteins that can be decarboxylated in response to the BME treatment were identified using mass spectrometry. See Supplemental Figure S3 for the locations of carbonylated protein spots (CPS) in 2-dimensional gels. C.I. refers to confidence interval, which indicates the reliability of an estimate of mass spectrometry analysis.