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Sulfonation of the resolving cysteine in human peroxiredoxin 1: A comprehensive analysis by mass spectrometry

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ABSTRACT

Peroxiredoxin 1 (Prx1) is an essential peroxidase that reduces cellular peroxides. It holds 2 indispensable cysteines for its activity: a peroxidatic cysteine (C_P) for peroxide reduction and a resolving cysteine (C_R) for C_P regeneration. C_P can be readily sulfonated to C_P–SO_3H by protracted oxidative stress, which inactivates Prx1 as a peroxidase. By comparison, sulfonation of C_R to C_R–SO_3H in mammalian cells has only been reported once. The rare report of C_R sulfonation prompts the following questions: "can C_R–SO_3H be detected more readily with the current high sensitivity mass spectrometers (MS)?" and "do C_P and C_R have distinct propensities to sulfonation?" Answers to these questions could shed light on how differential sulfonation of C_P and C_R regulates Prx1 functions in cells. We used a sensitive Orbitrap MS to analyze both basal and H_2O_2-induced sulfonation of C_R and C_P in either recombinant human Prx1 (rPrx1) or HeLa cell Prx1 (cPrx1). In the Orbitrap MS, we optimized both collision-induced dissociation and higher-energy collisional dissociation methods to improve the analytical sensitivity of cysteine sulfonation. In the basal states without added H_2O_2, both C_P and C_R were partially sulfonated in either rPrx1 or cPrx1. Still, exogenous H_2O_2 heightened the sulfonation levels of both C_P and C_R by ~200–700%. Titration with H_2O_2 revealed that C_P and C_R possessed distinct propensities to sulfonation. This surprising discovery of prevalent Prx1 C_R sulfonation affords a motivation for future investigation of its precise functions in cellular stress response.

1. Introduction

Peroxiredoxins (Prxs) are potent antioxidant proteins. Understanding how their functions are regulated is important for managing stress-induced diseases. The antioxidant activity of some Prxs depends on two conserved cysteines: a peroxidatic cysteine (C_P) for peroxide reduction and a resolving cysteine (C_R) for C_P regeneration. In this study, we've optimized a mass spectrometry (MS) method to analyze cysteine sulfonation in Prx1, and to demonstrate that besides the familiar sulfonation of C_P, sulfonation of C_R is a physiological phenomenon that occurs in select cells. Plausible significance of C_R sulfonation in regulating the plethora of Prx1 functions to counter oxidative stress is discussed.

Prxs reduce peroxides at the expense of the free thiol on C_P (C_P-SH), whose oxidation - proton loss - has to be replenished by thioredoxins, before Prxs can reenlist in another cycle of peroxidase catalysis. Different classes of Prxs use distinct mechanisms to replenish C_P protons and renew Prxs' readiness to combat peroxides. Prx1 is a member of the 2-Cys classes of Prxs, whose C_P (C52 in human Prx1) interacts with C_R (C173 in human Prx1) on two antiparallel-facing Prx1 molecules. Within physiological peroxide concentrations, Prx1 attacks the O-O bond in a peroxide substrate, while sacrifices C_P-SH into a sulfenic acid (C_P-SOH, Supplemental Fig. S1, scheme I) [1–5]. C_P-SOH, in turn, can conjugate with the free thiol on the C_R (C_R-SH) of a neighboring Prx1 molecule, to form a disulfide-linked homodimer (Supplemental Fig. S1, scheme II). This C_P-C_R intermolecular disulfide can then be specifically reduced by thioredoxin 1 (Trx1, Supplemental Fig. S1, scheme III), returning C_P to a catalytically active C_P-SH. Alternatively, with elevated

Abbreviations: ACN, Acetonitrile; CBB, Coomassie brilliant blue; C_P, Peroxidatic cysteine, C52 in human Prx1; C_P-SH, Peroxidatic cysteine with free thiol; C_P-SOH, Peroxidatic cysteine with sulfenic acid; C_P-SO_2H, Peroxidatic cysteine with sulfinic acid; C_P-SO_3H, Peroxidatic cysteine with sulfonic acid; C_R, Resolving cysteine, C173 in human Prx1; C_R-SH, Resolving cysteine with free thiol; C_R-SO_3H, Resolving cysteine with sulfonic acid; CID, Collision-induced dissociation; HCD, Higher-energy collisional dissociation; IAA, Iodoacetamide; LC, Liquid chromatography; MS, Mass spectrometry; NCE, Normalized collision energy; NEM, N-ethylmaleimide; Prx1, Peroxiredoxin 1; Prxs, Peroxiredoxins; rPrx1, Recombinant human Prx1; cPrx1, Prx1 present in cells; PTM, post-translational modification; Srx, Sulfiredoxin; Trx1, Thioredoxin 1; XIC, Extracted ion chromatogram

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oxidative stress, C_P-SOH can be progressively over-oxidized to either a sulfinic acid (C_P-SO_2H) or sulfonic acid (C_P-SO_3H) (Supplemental Fig. S1, schemes IV–V). Overoxidation of Prx1 prevents the formation of the C_P-C_R intermolecular disulfide, thus hinders Prx1 reactivation by Trx1 and inactivates its peroxidase activity [1,4–8]. Fortuitously, sulfiredoxin (Srx) and related enzymes can reduce C_P-SO_2H to C_P-SOH and pave the path for Trx1 to reactivate Prx1 [9–12]. By comparison, overoxidation of C_P-SH to C_P-SO_3H has long been characterized as a mechanism for the permanent inactivation of Prx1 as a peroxidase, which may lead to its demise via proteolytic degradations [13,14].

Two main methods for analyzing Prx sulfonation are Western blotting using an anti-Prx-SO_2/SO_3 antibody [9,10] and MS, following gel electrophoresis or liquid chromatography (LC) separations of either recombinant Prxs [15,16], Prxs isolated from cell lines [11,12,14], blood or tissues [14,17,18]. Rabillound *et al.* used a Finnigan TSQ7000 MS to analyze Prx2, a member of the 2-Cys Prxs, and a close relative to Prx1 [17], and revealed for the first time of C_P-SO_3H in a Prx. The same group also used a matrix-assisted laser desorption/ionization MS in the negative ion mode, to discover that C_P-SH in Prx2 isolated from HeLa cells could be oxidized to C_P-SO_2H in addition to C_P-SO_3H [11]. Remarkably, they further identified C_P-SO_3H in both Prx1 and Prx3 in the same study, articulating a general mechanism for the irreversible inactivation of Prxs by sulfonation. These pioneering studies exemplified standard MS approaches to identify Prx sulfonation in additional species. For example, using both an antibody against C_P–SO_3H from a yeast Prx (Tsa1p) and a QSTAR pulsar QTOF MS, Lim *et al.* discovered that C_P sulfonation is the driver for switching Tsa1p from a peroxidase to a molecular chaperon, a phenomenon that exists for Prxs in many other species [3]. Based on the frequent observations of C_P–SO_3H, a general 2-Cys Prx [19,20] catalytic model has been proposed wherein only C_P, but not C_R, is sulfonated by excessive oxidation (Supplemental Fig. S1. See review [21]).

Our interest in studying Prx1 sulfonation stemmed from a serendipitous discovery of Trx1-directed S-nitrosation of Prx1, another redox post-translational modification (PTM), which can attenuate H_2O_2-induced sulfonation of Prx1 [5]. In order to confirm that S-nitrosation can indeed mitigate C_P–SO_3H formation in Prx1, we used an Orbitrap MS to map the sulfonation sites in rPrx1. Unexpectedly, we detected astonishingly high levels of C_R–SO_3H (data shown below). Through a thorough literature search, we discovered only a single report of using an Orbitap XL MS to discover C_R–SO_3H in Prx1 isolated from the livers of rats suffering from ischemia reperfusion injuries [18]. In fact, C_R–SO_3H has also been detected in a rapeseed 2-Cys Prx [16]. Here, we set out to determine whether C_R–SO_3H is indeed a reproducible physiological phenomenon. To this end, we optimized the Orbitrap MS parameters and compared the relative efficiencies of collision-induced dissociation (CID) and higher-energy collisional dissociation (HCD) for mapping C_R–SO_3H in Prx1-derived tryptic peptides. With an optimized MS method, we comprehensively compared whether C_P and C_R have different propensities to sulfonation, and assessed whether C_R sulfonation occurs in assorted cell types. Since diverse PTMs on Prx1 have been correlated with its diverse functions in cells and organisms [22, 23], we will discuss how differential sulfonation of C_P and C_R may regulate the diverse Prx1 functions in handling cellular stress responses.

2. Materials and methods

2.1. Materials

Purified recombinant full-length human Prx1 protein (rPrx1) and all antibodies used in this study were from Abcam (Cambridge, MA, USA). All chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated. MS-grade trypsin was purchased from Thermo Fisher Scientific (Waltham, MA USA). LC-grade acetonitrile (ACN) and water were obtained from T. J. Baker (Center Valley, PA, USA). HeLa cells, human neuroblastoma SH-SY5Y cells, human embryonic kidney 293 (HEK293) cells, and human fibroblast GM08680 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in growth media according to ATCC's instructions, and HL-1 cells were cultured in Claycomb Medium (Sigma-Aldrich, St. Louis, MO, USA). All media were supplemented with 10% FBS and penicillin/streptomycin at 37 °C and 5% CO_2.

2.2. Oxidation and analysis of rPrx1

Twenty-five μg of rPrx1 were initially reduced with 100 mM DTT at 37 °C for 1 h to obtain the reduced rPrx1. The ensuing proteins were precipitated using ice-cold acetone to free them from DTT. Reduced rPrx1 was then oxidized with 0–100 mM H_2O_2 at 37 °C for 30 min, followed by acetone precipitation to remove H_2O_2. The resulting proteins were analyzed by either reducing or non-reducing SDS-PAGE gels. Two hundred ng of the gel-resolved rPrx1 from each sample were analyzed by Western blotting to detect both total and sulfonated Prx1 (see methods below). The remaining proteins were purified by SDS-PAGE and stained with Coomassie Brilliant Blue (CBB) for in-gel digestion and sulfonation-site identification by LC/MS/MS described below.

2.3. Cell culture and induction of oxidative stress in cells

HeLa cells were grown in 10 cm plates at 37 °C in Dulbecco's modified Eagle's medium. The confluent cells were treated with either 1 mM H_2O_2 or media - as a control for 30 min. The cells were then harvested via centrifugation at 500 × *g* for 5 min and washed with PBS prior to SDS-PAGE and MS analyses. To analyze Prx1 sulfonation in other cells, SH-SY5Y cells, HEK293 cells, GM08680 cells, and HL-1 cells were also treated with or without H_2O_2 and analyzed as described for the HeLa cells.

2.4. Western blotting

For Western blotting analysis of rPrx1, 200 ng of rPrx1 in each sample were analyzed by both reducing and non-reducing gels and transferred onto the nitrocellulose membranes. Non-specific binding sites on the membranes were blocked with 5% milk and Prx1 were probed with either an anti-Prx1 (1:5000) or an anti-Prx-SO3 (1:2500) antibody. For Western blotting of Prx1 in HeLa cells (cPrx1), proteins were extracted using a lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA and pH 7.4), supplemented with 0.5% protease inhibitor cocktail (P8340, Sigma-Aldrich). The protein concentrations were measured using a Bradford assay kit (#5000201; BioRad, Hercules, CA, USA). Sixty μg of proteins from each cell extract were separated using a 12% reducing SDS-PAGE gel, transferred onto a nitrocellulose membrane and probed with the antibodies as described above.

2.5. In-gel digestion

Fifty μg of the proteins extracted from each cell treatment that contains cPrx1, or 1 μg/sample of rPrx1 protein were separated by SDS gels and the Prx1 bands in CBB-stained gels that migrated to the same positions as the Prx-SO_3 signals obtained from the Western blotting were excised for in-gel digestion. For the proteins in each gel band, the disulfide bonds were reduced using 100 μl of 10 mM DTT at 37 °C for 30 min. After the removal of DTT, the free thiols were alkylated with 55 mM of either iodoacetamide (IAA, pH 8.5) or N-ethylmaleimide (NEM, pH 7.2) at 37 °C for 30 min. After alkylation, the gel species were washed 3 times with 100 μl of 30% ACN in 25 mM NH_4HCO_3 and dehydrated successively using 80, 60 and 40 μl of 80% ACN for 10 min each. To digest the proteins in each sample, 30 μl of 10 ng/μl trypsin in 25 mM NH_4HCO_3 (pH 8.3) were first added and incubated at 37 °C for

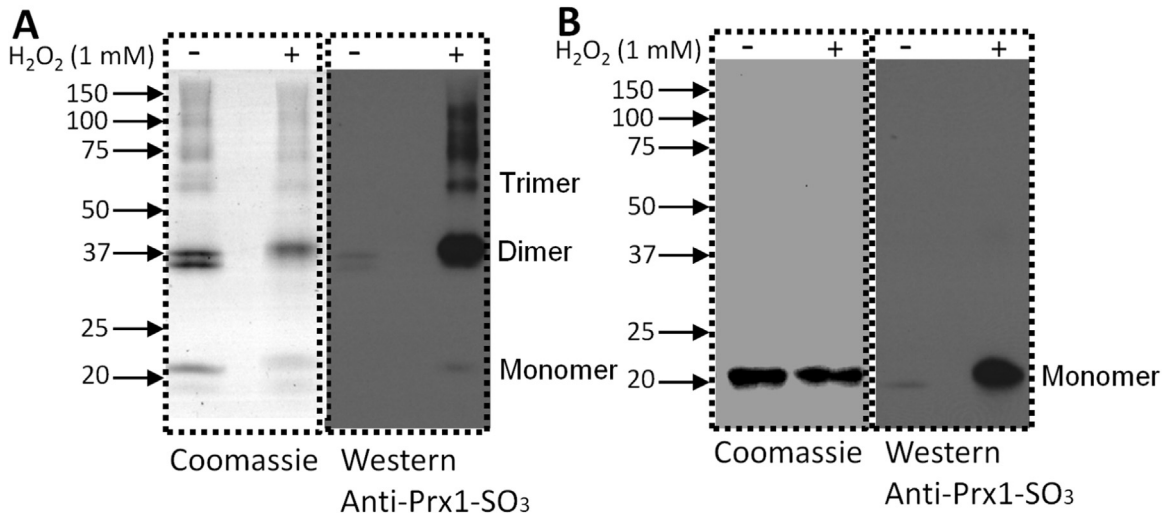

Fig. 1. SDS-PAGE and Western blotting of sulfonated rPrx1 following H_2O_2 treatment. rPrx1 was reduced with 100 mM DTT for 1 h at 37 °C. After acetone precipitation to remove the DTT, reduced rPrx1 was treated with or without 1 mM of H_2O_2. The resulting proteins were separated using either non-reducing (A) or reducing (B) 12% SDS PAGE gels. Two hundred ng of rPrx1 in each sample was analyzed by Western blotting with an anti-Prx-SO3 antibody.

30 min, and then sufficient 25 mM NH_4HCO_3 were added to cover all the gel pieces. The reactions were carried out at 37 °C overnight. Tryptic peptides were extracted for 3 times, using 60 µl of 1% trifluoroacetic acid for 10 min each, and for 3 more times with 80% ACN for 10 min each. The pooled peptide solutions were dried to ~10 µl with a SpeedVac (Eppendorf North America, Hauppauge, NY, USA). The peptides were desalted using the Pierce C_{18} spin columns according to the manufacturer's protocol (Thermo Fisher Scientific).

2.6. LC/MS/MS

The desalted peptides were analyzed on an LTQ Orbitrap Velos mass spectrometer coupled with a Dinoex Ultimate 3000 RSLC nano LC system through a Proxeon nano-electrospray ion source (Thermo Fisher Scientific). The peptides were separated by a RPLC capillary column (75 µm ×150 mm, 3 µm, 100 Å, C_{18}, CMP Scientific, Brooklyn NY, USA) at 250 nl/min using a 85-min gradient (Solvent A: 2% ACN, 0.1% formic acid, Solvent B: 85% ACN and 0.1% formic acid), which included a 45-min gradient from 3% B to 30% B, followed by a 5-min gradient from 30% B to 50% B, and another 5-min gradient from 50% B to 95% B. The eluted peptides were directly introduced into the MS with a spray voltage of 2.2 kV and a capillary temperature of 275 °C. The MS spectra were acquired in the positive ion mode using a data-dependent method, where after each full MS scan, the ten most intense peptide ions with multiple-charge states were fragmented using either CID or HCD, with a 60-sec dynamic exclusion window. To ensure that peptides containing C_P and C_R in Prx1 were selected for MS/MS analysis, the m/z values of 2^+, 3^+, and 4^+ of both sulfonated and carbamidomethylated (or NEM-alkylated) tryptic peptides were specified in an inclusion list for targeted fragmentation. The MS1 mass range was set from m/z 300 to 1650 at a resolution of 60,000 at FWHM, with the lock mass option enabled. For MS/MS peptide fragmentation, the normalized collision energies (NCE) were evaluated between 24% and 40% for both CID and HCD. An optimized NCE of 32% in CID mode was later used to acquire all the biological data in this study.

2.7. Database search

Protein database searches were performed with the Proteome Discoverer software (Thermo Fisher Scientific, version 1.4.12). The LC/MS/MS spectra were searched against a Swissprot human or rat protein sequence database (downloaded in July 9th, 2016, with 20,255 human or 7724 rat entries), using a local MASCOT search engine (V.2.4). The search parameters were set as the following: trypsin with 2 missed cleavage sites; precursor mass tolerance was set at 10 ppm and the fragment mass tolerance was set at 0.5 Da for CID and 0.1 Da for HCD. Oxidation of methionine, sulfenation, sulfination, sufonation and carbamidomethylation of cysteine were set as variable modifications. Both protein and peptide False Discovery Rates (FDR) were filtered to less than 1%.

2.8. MS quantification and statistical analysis

For peptide quantification, MS1 extracted ion chromatograms (XIC) for the selected peptides were obtained with the MS1 Full-Scan Filtering Module of the Skyline Software (v3.5) (https://skyline.gs.washington.edu/). Briefly, a spectral library was first created from all the raw LC/MS/MS data. A Prx1 FASTA file was imported to obtain the XIC for all Prx1 peptides identified, including the sulfonated peptides. The MS scans within two minutes of the LC retention times from each MS/MS spectrum that matched to a given peptide were included for the quantification. For each peptide, up to 3 ion charge states (2^+, 3^+, and 4^+) were summed for quantification. For each ion, up to 3 isotope peaks (M, M + 1, and M + 2, at a resolution of 60,000) were used for XIC integration. Following the Skyline analysis, the XIC of the C_P- or C_R-sulfonated peptides were confirmed manually by the retention time, charge states and MS/MS spectra. The XIC signals for the sulfonated peptides were normalized to the combined XIC signals of all the Prx1peptides identified in that LC/MS/MS run. In each experiment series, sulfonation change was calculated as the fold change in percentage (%) over one of the controls with no added H_2O_2 oxidation, whose value was set as 100%. ImageJ software was used for the quantificaiton of the Western blotting results. Three biological repeats were conducted for all experiments, unless noted otherwise. The changes were considered statistically significant based on the Student's *T*-test, $p < 0.05$.

3. Results

3.1. Western blotting of differential sulfonation of rPrx1 oligomers

In order to identify the sulfonation sites in Prx1, we first examined the levels of sulfonation by Western blotting of rPrx1 oxidized with 1 mM H_2O_2. As expected, the sulfonation levels of rPrx1 were dramatically increased after H_2O_2 treatments (Fig. 1). Besides being a peroxidase, Prx1 is also a protein-folding chaperon; an activity can be

induced by its oligomerization following oxidation [22,24]. To detect H_2O_2-induced sulfonation changes among the different rPrx1 oligomers linked by disulfides, we also compared the results of both non-reducing and reducing gels to separate the disulfide-linked oligomers for the Western blot analyses. The resulting blots revealed that after the H_2O_2 treatments, rPrx1 dimers contained the highest sulfonation level compared to those in the other rPrx1 oligomers and the monomer. Likewise, hydrogen peroxide also induced dramatic sulfonation in rPrx1 tri-mer to high mass oligomers; yet surprisingly little sulfonation occurred in rPrx1 monomer (Fig. 1A, right panel). In order to comprehensively map the sulfonation sites by LC/MS/MS in all rPrx1 oligomeric states, we reduced all the rPrx1 oligomers to a monomer and attained a single H_2O_2-induced sulfonation band in the Western blot (Fig. 1B).

3.2. Optimization of a LC/MS/MS method to map Prx1 sulfonation

In order to obtain optimal CID and HCD MS/MS spectra for sulfonated cysteines, we tested five different NCEs −24%, 28%, 32%, 36%, and 40% - to evaluate their impact on detecting C_R–SO_3H in a rPrx1 tryptic peptide (amino acids 169–190). Spectra obtained from an Orbitrap Velos MS showed that both CID and HCD approaches were effective for identifying C_R-SO_3H (Supplemental Figs. S2 & S3). Compared to HCD, CID was more robust at detecting the b^4 and b^5 and y^{17} and y^{18} MS/MS ions surrounding C_R-SO_3H, with a wider range of NCEs of 28–40% effective for locating C_R-SO_3H (Supplemental Figs. S2A-E compared to Supplemental Figs. S3A-E). The best NCE in CID for both detecting C_R-SO_3H and obtaining rich y- and b-series of ions for peptide identification was 32% (Supplemental Fig. S2C). By comparison, the best NCE in HCD for detecting C_R-SO_3H was at 28% (Supplemental Fig. S3B), with other NCEs produced less b^4 and b^5 and y^{17} and y^{18} ions that are necessary for the unambiguous mapping of C_R-SO_3H (Supplemental Fig. S3).

Besides C_R-SO_3H, 32% NCE in CID was also effective for mapping C_P-SO_3H (Supplemental Fig. S4 & Fig. 2). However, none of the NCEs in HCD tested could provide an informative MS/MS spectrum for mapping C_P-SO_3, indicating that HCD was inferior to CID for this study. In lieu of these observations, all subsequent data were obtained in the CID mode at 32% NCE. Representative MS/MS spectra in Figs. 2 and 3 demonstrate that both C_P and C_R were partially sulfonated under H_2O_2 treatments, with the remaining cysteine free thiols alkylated by IAA.

3.3. Basal and H_2O_2-induced sulfonation of C_P and C_R in rPrx1

From the Skyline MS1-based quantitative analyses, basal sulfonation can be detected at both C_P and C_R in rPrx1, even without H_2O_2. As expected, a 30-min H_2O_2 treatment at 1 mM significantly boosted rPrx1 sulfonation at both C_P to ~300% and C_R to ~670%, when compared to the 100% basal levels (Fig. 4). Remarkably, H_2O_2 more than doubled the increase of the sulfonation at C_R at 670%, over C_P at 300% (Fig. 4).

Since C_R-SO_3H is a relatively new PTM of Prx1, it is important to determine whether this PTM could simply be an artifact from the in-gel digestion, especially during DTT reduction and subsequent IAA alkylation. Thus, we evaluated whether NEM, an alkylating reagent that is more efficient than IAA [25], can diminish the LC/MS/MS detection of C_R-SO_3H. Still, with identical H_2O_2 treatments, we observed the opposite: ~670% sulfonation induction with IAA and ~900% with NEM (Supplemental Fig. S5).

Given the new evidence of C_R-SO_3H, we performed an H_2O_2 titration to ascertain whether first, C_R and C_P have similar propensities to sulfonation and second, whether the commonly used PrxSO3 antibody can detect the sulfonation of both C_P and C_R equally. Western blotting confirmed the anticipated increase of PrxSO3 from escalating H_2O_2 (Supplemental Fig. S6A). We then plotted the Western blotting titration curve along with the LC/MS curves for the tryptic peptides with either C_P-SO_3H or C_R-SO_3H (Supplemental Fig. S6B). Since the LC/MS curve for C_P-SO_3H, instead of C_R-SO_3H, aligns better with the Western blotting curve, we conclude that this antibody mostly reacts with the C_P-SO_3H epitope. From the LC/MS titration curves, at 1 mM H_2O_2, C_R-SO_3H signals were higher than those of C_P-SO_3H. By comparison, C_P-SO_3H was indeed superior to C_R-SO_3H, only when being induced at a pharmacological concentration of 100 mM H_2O_2. Thus, C_P and C_R appear to have distinct propensities to H_2O_2-induced sulfonation.

3.4. Basal and H_2O_2-induced sulfonation of C_P and C_R in cPrx1

In order to confirm that C_R sulfonation can also occur in cells, we treated the cultured HeLa cells with either the media control or supplemented with 1 mM H_2O_2 for 30 min and analyzed PrxSO$_3$H by Western blotting. Unexpectedly, we readily observed basal Prx1-SO_3H in the unoxidized HeLa cells (Fig. 5A). As expected, we saw significantly elevated Prx1-SO_3H after the H_2O_2 treatment (Fig. 5B). We quantified both C_P-SO_3H and C_R-SO_3H-containing peptides by LC/MS/MS (Fig. 6). Thirty-minute H_2O_2 treatments significantly raised C_P-SO_3H to ~200% and C_R-SO_3H to ~400% of their respective control peptides in the unoxidized cells (Fig. 6).

3.5. C_P and C_R sulfonation in other cells

So as to examine whether C_R-SO_3H is a widespread phenomenon in other cells, we also used LC/MS/MS to examine both C_P-SO_3H and C_R-SO_3H in cPrx1 of 4 other cell types. Both HEK293 and GM08680 cells behaved like HeLa cells. Without the added H_2O_2, basal C_P-SO_3H and C_R-SO_3H occurred in cPrx1 (Supplemental Table S1). With H_2O_2 added, both C_P-SO_3H and C_R-SO_3H were elevated. On the other hand, SH-SY5Y cells contained only basal C_R-SO_3H, which was increased by H_2O_2. Surprisingly, SH-SY5Y cells had no detectable C_P-SO_3H, even with added H_2O_2. Still, HL-1 cells were the most remarkable, regardless of H_2O_2; they contained no measureable sulfonation at either site in Prx1; instead, HL-1 cells had abundant IAA-modified C_P and C_R in Prx1 (not shown).

4. Discussion

Prx1 is an enigmatic protein with assorted cellular functions. Besides its “day job” as a guardian against oxidative stress, it is also a jack of other fascinating trades [26]. For example, oxidation-induced oligomerization can transform Prx1 into a molecular chaperone to preserve the proper folding in the midst of cellular stress [20,22]. Furthermore, blood circulations of sulfonated Prx1 in animal models synchronize with their circadian rhythms [27], making it a rare molecular marker for biological clocks and sleep cycles. The ability of Prx1 to play distinct roles is predicated, in part, on its ability to wear different hats – different PTMs – on evolutionarily conserved residues. In addition to serine phosphorylation and lysine acetylation [28,29], numerous PTMs have been discovered on the four conserved cysteines in Prx1 [30,31]. Among them, progressive oxidation of C_P is the best studied in the context of the peroxidase enzymatic mechanism of Prx1. Those PTMs include sulfenation (-SOH), sulfination (-SO_2H) and sulfonation (-SO_3H) [2,3,8,32]. Apart from C_P, partial oxidation of C_R has also been observed [4], whose PTM functions are largely unexplored. In this study, we aim to demonstrate that C_R-SO_3H is an over looked PTM of Prx1, which may play important roles in regulating some of the diverse functions of Prx1.

We have shown that sulfonation of C_R is a reproducible phenomenon that occurs readily in several cells, by capitalizing on Orbitrap's fast MS/MS scan speed. From our experience, MS/MS fragmentations of the C_R-SO_3H-containing peptide ions were not as efficient as those with C_P-SO_3H (Figs. 2 & 3), which could account for the rare detection of C_R-SO_3H in previous studies. This technical challenge can be overcome with proper optimization of the NCEs and other relevant MS/MS

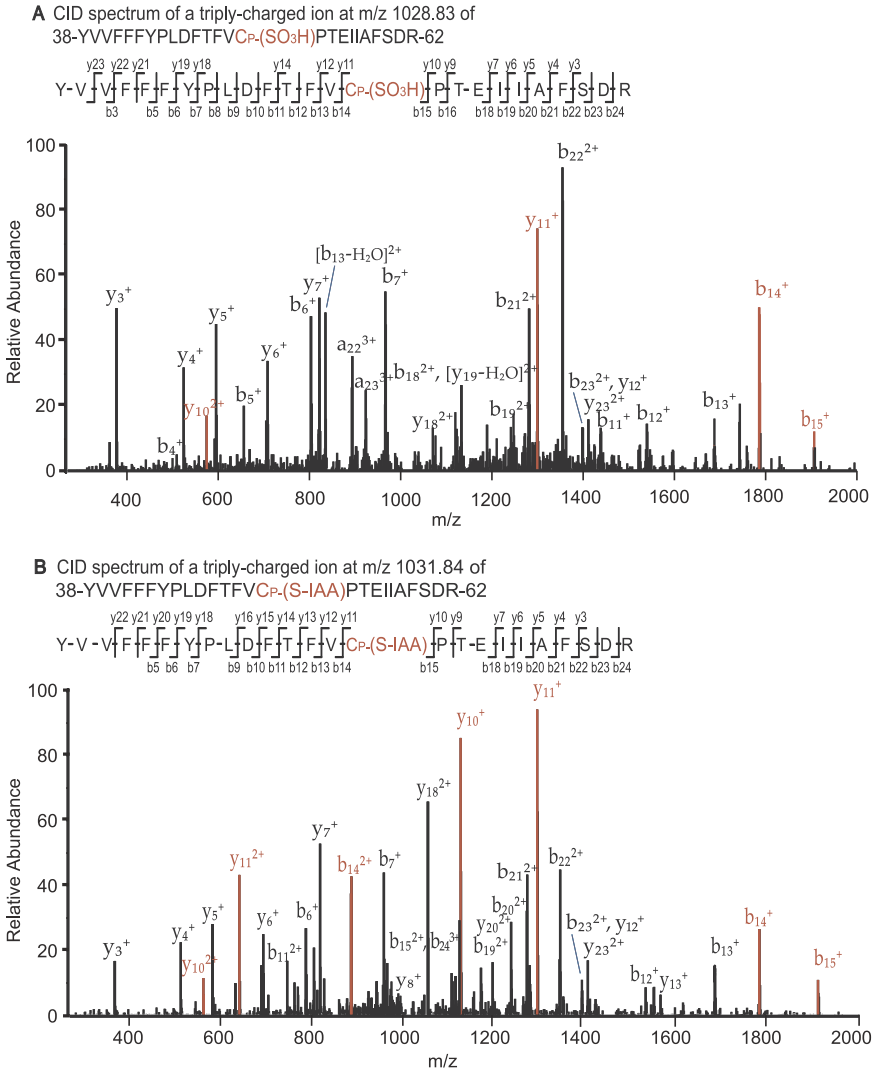

Fig. 2. CID spectra of a Prx1 peptide (amino acids 38–62) with C_P-SO_3H or C_P alkylation. (A) An MS/MS spectrum of a $3H^+$ ion at *m/z* 1028.83 for the sulfonated peptide. (B) An MS/MS spectrum of a $3H^+$ ion at *m/z* 1031.84 for the alkylated peptide. Sulfonated rPrx1 was obtained from the reducing gel outlined in Fig. 1. The strings of b- and y-series ions from the MS/MS spectra matched to 38-VVFFFYPLDFTFVCPTEIIAFSDR-62 in human Prx1. The sulfonated peptide with a Cys +47.98 amu was identified with a Mascot score of 51 (A). The alkylated peptide with a Cys +57.02 amu was identified with a Mascot score of 70 (B). The PTMs were found between b^{14} and b^{15} and between y^{10} and y^{11} ions in each spectrum (marked red), which confirmed the sulfonation and alkylation of C_P in rPrx 1 (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

conditions. With an optimized MS method, we've been able to comprehensively analyze Prx1 sulfonation in both purified rPrx1 and cellular cPrx1, and under both basal condition and H_2O_2-induced oxidation. To benchmark the significance of C_R sulfonation, we also compared C_R-SO_3H with C_P-SO_3H, whose biology has been well understood from the work of the pioneers in Prxs [9,10,12].

The first unusual finding from this study is the basal sulfonation of C_R and C_P in both rPrx1 and cPrx1. Conceivably, these observations could be either biological phenomena or analytical artifacts. Although it is impossible to eliminate cysteine oxidation during sample processing, our data on the diverse cPrx1 sulfonation patterns support the notion that basal sulfonation is a physiological phenomenon; from identical analytical procedures, we didn't see basal sulfonation in all cells. Without added oxidation, we detected sulfonation at both C_P and C_R only in HeLa, HEK293 and human fibroblasts GM08680 cells, sulfonation only at C_R in human neuroblastoma SH-SY5Y cells, and no sulfonation at either C_P or C_R in HL-1 cells (Supplemental Table S1). Similar to cPrx1, basal sulfonation of rPrx1 is likely derived from the expression cell systems before rPrx1 purification. If O_2 in the test tubes drove bulk of the detected sulfonation, we would have expected to see more basal sulfonation in rPrx1 than in cPrx1, since rPrx1 would have much longer time to react with O_2 during purification and storage. Yet, we observed the opposite (data not shown). Thus, although we cannot completely eliminate analytical artifacts, their contribution to basal sulfonation is likely negligible. Overall, basal sulfonation of both C_P and C_R in Prx1 appears to be physiological phenomena in diverse, albeit not all cell types.

Basal sulfonation of a fraction of C_P and C_R in some but not all cellular Prx1 could be attributed, in part, to the diverse cellular mechanisms that regulate Prx1 oxidation, thus diverse signaling functions. For example, Trx1, glutathione reductase and Srx can reduce select oxidative PTMs on Prx1 and, in turn may indirectly reduce basal sulfonation of C_P and C_R. Since we hypothesize that basal sulfonation is likely physiological, a fraction of Prx1 is probably inactivated as a peroxidase in untreated cells, either due to the direct drop in C_P availability or indirectly via the lack of C_R for C_P regeneration (Supplemental Fig. S1). Yet, even inactivated as a peroxidase, Prx1 may hold other biological functions. For example, sulfonation could activate Prx1 as a protein-folding chaperon and other signaling functions. Then the inactivation of Prx1's peroxidase activity is a simple trade-off of one activity for another. This trade-off appears to hold true for other Prxs. Si *et al.* reported that an atypical 2-Cys Prx in *C. glutamicum* mainly exists as a monomer and displays a Trx-dependent peroxidase activity at low peroxide concentrations; over oxidation

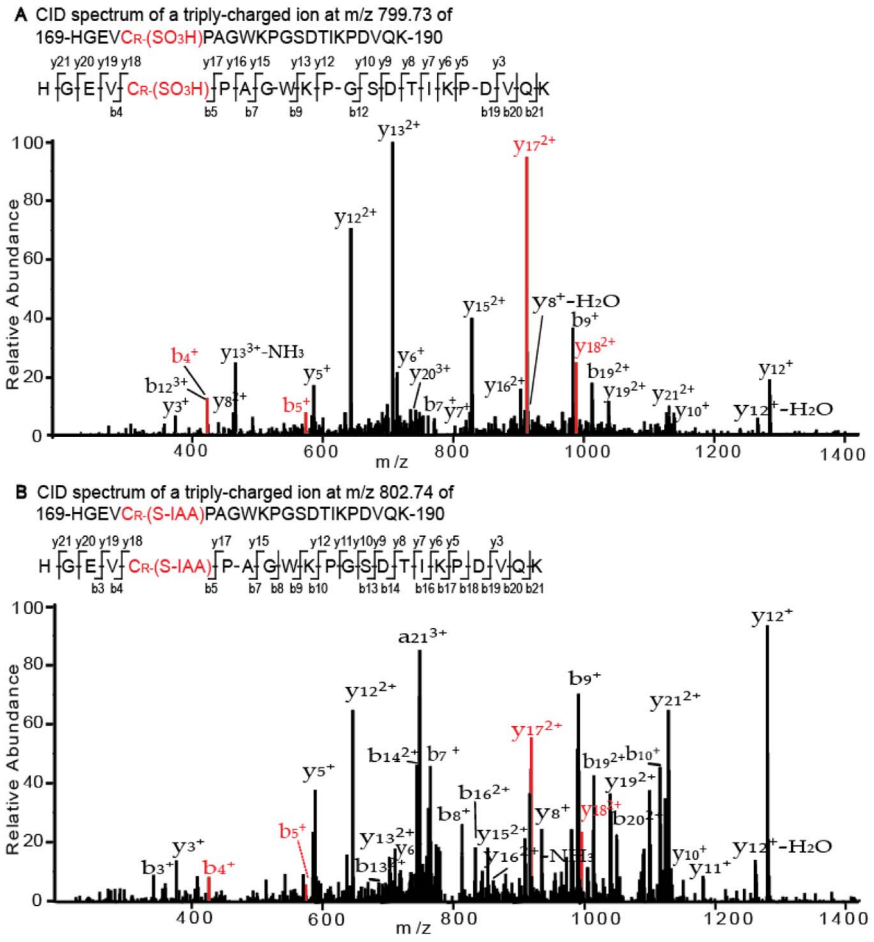

Fig. 3. CID spectra of a Prx1 peptide (amino acids 169–190) with C_R-SO_3H or C_R alkylation. (A) An MS/MS spectrum of a $3H^+$ ion at *m/z* 799.73 for the sulfonated peptide. (B) An MS/MS spectrum of a $3H^+$ ion at *m/z* 802.74 for the alkylated peptide. Sulfonated rPrx1 was obtained from the reducing gel outlined in Fig. 1. The strings of b- and y-series ions from the MS/MS spectra matched to 169-HGEVCPAGWKPGSDTIKPDVQK-190 in human Prx1. The sulfonated peptide with a Cys +47.98 amu was identified with a Mascot score of 52 (A). The alkylated peptide with a Cys +57.02 amu was identified with a Mascot score of 68 (B). The PTMs were found between b^4 and b^5 and between y^{17} and y^{18} ions in each spectrum (marked red), which confirmed the sulfonation and alkylation of C_R in rPrx 1 (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

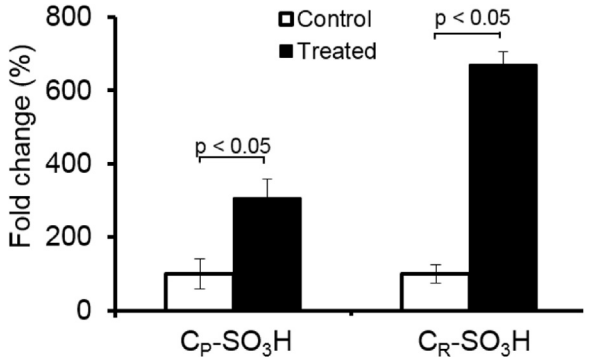

Fig. 4. Comparison of H_2O_2 induction of C_P–SO_3H and C_R–SO_3H in rPrx1. Following a 30-min and 1 mM H_2O_2 treatment, sulfonated rPrx1 was obtained from the reducing gels outlined in Fig. 1 and analyzed by LC/MS/MS as described in Section 2. XIC for the precursor ions that matched to the peptides containing either C_P-SO_3H or C_R-SO_3H were obtained by Skyline. The XIC signals for the sulfonated peptides were normalized to the combined XIC of all the identified rPrx1peptide ions. Sulfonation change is presented here as the fold change (%) over a control with no added H_2O_2 oxidation, whose value is set as 100%. The changes were considered statistically significant based on the Student's *T*-test (N = 3/group).

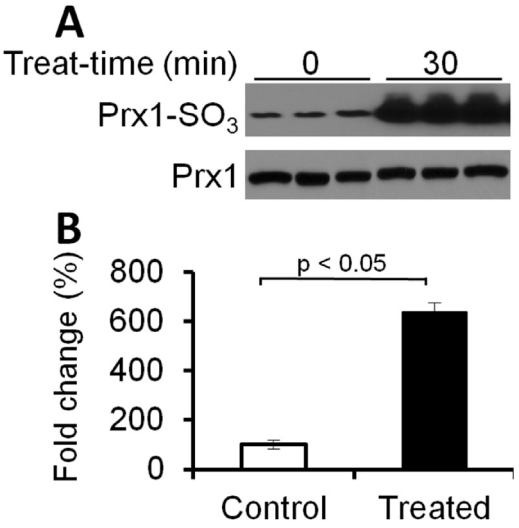

Fig. 5. Western blots for Prx1-SO_3 in HeLa cells. Cells were treated for 30 min with either media alone or supplemented with 1 mM of H_2O_2. Sixty μg of proteins from each sample were separated using a 12% reducing SDS-PAGE gel. (A) An anti-Prx1-SO_3 antibody (Abcam, 1:2500 dilution) was used to detect Prx1-SO_3. Total Prx1 was detected using an anti-Prx1 antibody. (B) Substantial basal Prx1-SO3 was readily observed in the untreated cells and significantly increased Prx1-SO3 was detected after the H_2O_2 treatment (N = 3/group).

induces Prx sulfonation and irreversibly inactivates its peroxidase activity, and in turn, activates its oligomerization and chaperone activities [33]. Thus, the Yin and Yang relationship of the peroxidase and chaperone activity of Prx is crucial for maintaining protein home-

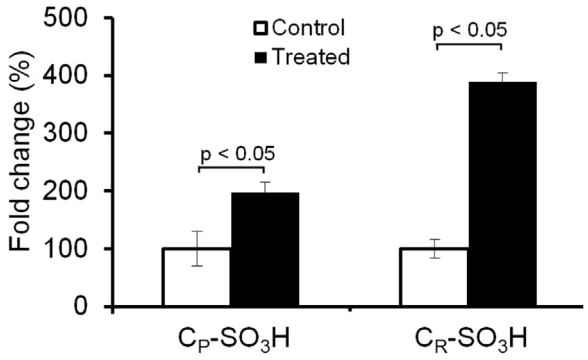

Fig. 6. Comparison of H_2O_2 induction of C_P–SO_3H and C_R–SO_3H in cPrx1. Following a 30-min and 1 mM H_2O_2 treatment of the HeLa cells, peptide sulfonation events were analyzed and quantified as described in Fig. 4 (N = 3/group).

ostasis in cells. The novel observation of basal Prx1 sulfonation suggests that some Prx1's chaperone or other signaling activities may already be primed for activation in cells with low oxidative stress, perhaps to prepare for counteracting anticipated escalation of oxidative stress in certain cells.

Besides basal sulfonation of Prx1, the second novel finding from this study is that C_R and C_P possess distinct propensity to H_2O_2-induced sulfonation. Overall, the LC/MS signals for peptides containing C_R-SO_3H were much stronger than those containing C_P-SO_3H (Supplemental Table S1). For example, in H_2O_2-treated SH-SY5Y cells, we observed no peptides containing C_P-SO_3H, but only the ones with C_R-SO_3H. Furthermore, from the LC/MS titration curves (Supplemental Fig. S6 & Fig. 6), 1 mM H_2O_2-induced C_R-SO_3H signals were higher than those of C_P-SO_3H, suggesting at modest concentrations, C_R reacted more efficiently than C_P with H_2O_2. By comparison, at a pharmacological concentration of 100 mM H_2O_2, C_P was far superior to C_R to become sulfonated, confirming it as the main catalytic cysteine for peroxide reduction by Prx1.

Based on the distinct propensities of C_P and C_R to H_2O_2 titration, we appraised whether a widely used rabbit polyclonal Prx-SO_3 antibody, which was developed against a C_P-based antigen (Abcam, ab16830), may also recognize C_R sulfonation with equal efficiency. From the H_2O_2 titration experiment, we plotted the Western blotting titration curve along with the LC/MS curves (Supplemental Fig. S6), and found that this antibody likely reacts more specifically with C_P-SO_3H. More conclusive determinations will require synthetic peptides containing either sulfonation sites, which is beyond the scope of this study. To accurately study the functions of C_R-SO_3H, a specific antibody targeting C_R-SO_3H is needed to delineate probable divergent functions for C_P and C_R besides their “day jobs” in the peroxidase cycle.

Since C_R sulfonation is a new phenomenon, we have paid extra attention to ensuring that this is not an analytical artifact, especially following H_2O_2 treatments. Since during sample handling, extended exposure of cysteine thiols to oxidants is likely a key factor for artificial as opposed to biological sulfonation, we’ve evaluated both alkylation timing and alkylating reagent types on the relative yield of C_R-SO_3H in rPrx1. If C_R-SO_3H was largely an analytical artifact, we would expect to see more of it with delayed alkylation or with a slower alkylating reagent. After the induction of rPrx1 sulfonation with 1 mM H_2O_2 for 5 min, we compared C_R-SO_3H levels between immediate IAA alkylation of proteins with delayed alkylation during in-gel digestion, and found no significant difference (data not shown). To go one-step further, we then compared the impact of conventional IAA and NEM, another alkylating reagent with purported higher alkylation efficiency than IAA [25], as the alkylation reagents for the in-gel digestions. Remarkably, we didn’t observe significant decrease of C_R-SO_3H from alkylation with NEM over IAA (Supplemental Fig. S5). We shall note that the alkylation reaction conditions were slightly different between IAA (pH 8.5) and NEM (pH 7.2), according to the manufactures’ datasheets. Thus H_2O_2-induction of C_R sulfonation is not an analytical artifact from alkylation.

The unusual findings of high level of C_R sulfonation lead to at least two implications: (1) Sulfonated C_R cannot form a disulfide with C_P; thus foils Trx1's ability to replenish protons at both C_P and C_R for another round of peroxidation reaction. Data from this study suggests that C_R is highly amenable to sulfonation in some, but not all cells, because they (e.g. HL-1 cardiac cells) may possess protective mechanisms to selectively attenuate the sulfonation of either C_R or C_P, thus preserving their free thiols in the continuation of the peroxidase cycle of Prx1, and (2) Sulfonation of C_R is likely a means to divert a portion of Prx1 away from the peroxidation cycles, for other possible redox signaling functions. For example, Prx1 can form dimers, decamers and even higher molecular weight oligomers [34]. From this study, we found that each Prx1 complex has different propensities to sulfonation (Fig. 1). Therefore, it is conceivable that C_R sulfonation may signal the partition of a portion of Prx1 away from the offensive actions against cellular peroxides, but to the defensive functions of maintaining the proper folding of key cellular proteins or other redox signaling functions.

With the novel findings in this study, we propose that both C_P and C_R in Prx1 can be oxidized to C_PSO_3H and C_RSO_3H (Supplemental Fig. S7). Compared to the well-known canonical mechanism for C_P sulfonation (Supplemental Fig. S7A), we propose here two alternative mechanisms for C_R sulfonation (Supplemental Fig. S7 B & C), under increasing oxidation in cells. In the canonical mechanism for progressive oxidative modifications of C_P, C_P-SH in a reduced Prx1 monomer becomes oxidized to C_P-SOH (I), which leads to the formation of a covalent Prx1 dimer (II). Trx1 restores this covalent Prx1 dimer back to the active and reduced Prx1 monomers (III). Too much peroxide produces C_P-SO_2H (IV) and C_P-SO_3H (V). Srx can reduce C_P-SO_2H back to C_P-SOH.

In the alternative mechanism 1 (Supplemental Fig. S7B), C_R-SH in reduced Prx1 monomer becomes oxidized to C_R-SOH (VI), which may also lead to the formation of the covalent Prx1 dimer (VII). Too much peroxide produces C_R-SO_2H (VIII) and C_R-SO_3H (IX). The alternative mechanism 1 for C_R-SO_3H formation does not preclude simultaneous oxidation of C_P; this model may also apply to non-covalent Prx1 dimers/oligomers. Since sulfonation appears to occur more prevalently in covalent Prx1 dimers and oligomers (Fig. 1A), we also proposed an alternative mechanism 2 (Supplemental Fig. S7C) to account for the sulfonation of C_R, in which too much peroxide produces an over-oxidized covalent Prx1 dimer, with C_R-SOH (X), subsequently C_R-SO_2H (XI) and C_R-SO_3H (XII). The alternative mechanism 2 for C_R-SO_3H formation does not preclude simultaneous oxidation of C_P; this model may also apply to covalent Prx1 oligomers. Ultimately, sulfonation of C_P and C_R may differentially regulate downstream signaling molecules to regulate the diverse biological functions of Prx1 [4,5].

5. Conclusion

In summary, C_R sulfonation is a robust physiological phenomenon in several but not all cells. Despite the vast knowledge on Prx1 and related molecules, there is still much to be learned about these evolutionarily conserved redox-signaling molecules. We report here that C_R sulfonation is readily induced by both chemical and cellular oxidative stress. Understanding the regulations C_R sulfonation can shed light on Prx1's diverse functions beyond peroxidase, and may lead to the discovery of novel redox signaling pathways.

Disclosures

The authors declare that they have no conflict of financial interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2017.04.341.

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