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The synthesis and analysis of S-nitorsylated paraoxonase 1

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ABSTRACT

Post-translational modification (PTM) of proteins plays a crucial role in health and disease by affecting numerous aspects of protein structure, function, stability and subcellular localization. Protein S-nitrosylation is one type of PTM that involves the covalent modification of cysteine sulfhydryl groups with nitric oxide (NO) and has a regulatory impact similar to phosphorylation. The enzyme paraoxonase 1 (PON1) is associated with high-density lipoprotein (HDL), and is responsible for many of HDL's antiatherogenic properties. The enzyme contains a free thiol group at Cys-284 which can also be modified covalently. As part of our effort to study the effect of PTMs on PON1 activities and properties and its implication for cardiovascular disease, we examined PON1's ability to undergo S-nitrosylation on its free Cys-284. Recombinant (re) PON1 was trans-S-nitrosylated by several NO donors, glutathione-NO (GSNO) was found to be the most effective. The S-nitrosylated rePON1 was analyzed by Q-TOF LC/MS and by Saville–Griess assay: the two analytical methods revealed closely similar results. rePON1 was also nitrosylated by nitrosylated human serum albumin (HSA-NO) via protein–protein trans-nitrosylation. HSA-NO transferred an NO group to rePON1 much more efficiently than GSNO with the formation of a higher than 70% rePON-NO when incubated with a 40-fold excess of a HSA-NO/HSA mixture. RePON1-NO was relatively stable: storage for 3 days at 37 °C resulted in only 25% decomposition. This is the first report of PON1's S-nitrosylation via GSNO and HSA-NO.

1. Introduction

Physical and biological properties of proteins can be changed by either their covalent or physical interactions with endogenous elements in their milieu. Covalent modifications of proteins after the translation step (post-translational modifications—PTMs), increase proteome diversity by 10- to 100-fold. PTM of proteins plays a central role in health and disease, affects protein structure and alters protein function, stability and subcellular localization [1–3]. Phosphorylation is the most prominent example of PTM of proteins, affecting their overall charge, inducing 3D structural changes and promoting protein–protein interactions [4]. However, recent studies have shown that PTM of proteins' cysteine thiols plays an important role in redox and cell signaling [5]. The thiol (-SH) moiety on the side chain of the amino acid cysteine is particularly sensitive to redox reactions and is, in fact, an established redox sensor [6,7]. Thiols can interact with a variety of endogenous elements to form reversible covalent modifications. S-nitrosylation of cysteine residues is another important PTM that regulates protein function and cell signaling. To date, thousands of proteins have been identified as potential S-nitrosoproteins (protein-SNOs) [8–11], which regulate protein activities either allosterically or by direct modification of an active cysteine site. S-nitrosylation may function as an important regulatory mechanism for protein activities within diverse cellular processes and biochemical pathways, including signal transduction, DNA repair, ion-channel regulation, and apoptosis [8,12,13].

PON1 is a calcium-dependent high-density lipoprotein (HDL)-bound enzyme. This enzyme catalyzes the hydrolysis of multiple compounds, such as organophosphates, aryl esters and lactones. Many of the antiatherogenic functions of HDL are attributed to its associated PON1 [14,15]. Epidemiological evidence demonstrates that low PON1 activity is associated with increased risk of cardiovascular events and disease [16,17]. PON1 contains a free thiol group at Cys-284 that is associated with its activity. In previous studies, we found that linoleic acid hydroperoxide (LA-OOH) present in the human carotid plaque inhibits PON1 activity via oxidation of its free thiol group [18] and Glabridin, an isoflavan isolated from licorice root prevent PON1 inhibition by LA-OOH [19].

As part of our effort to study the presence and properties of modified PON1 in human disease and its implications for atherosclerosis and cardiovascular disease, PON1's ability to undergo S-nitrosylation on its free Cys-284 was examined. PON1 was nitrosylated by various methods which can occur in vivo. The analytical

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methods used to identify and characterize S-nitrosylated PON1 are described, as are some of the properties of PON1-SNO.

2. Materials and methods

2.1. Materials

S-nitrosoglutathione (GSNO) (CAS; 57564-91-7), reduced glutathione (GSH) HEPES buffer (1 M), ethylenediaminetetraacetic acid (EDTA), neocuproine, dithiothreitol (DTT), mercury dichloride, sulfanilamide, *N*-(1-napthyl)ethylenediamine dihydrochloride and human serum albumin (HSA) (CAS 70024-90-7) were purchased from Sigma–Aldrich. All solvents were of HPLC grade.

2.2. Recombinant (re) PON1

RePON1 was generated in *Escherichia coli* by directed evolution as described previously [20].

2.3. S-nitrosylation of rePON1 using GSNO

RePON1 (62 μM) was dissolved in HEN buffer (50 mM HEPES buffer pH 7 containing 1 mM EDTA and 0.1 mM neocuproine). GSNO was added to the solution (final concentration 2.5 mM), and the solution was incubated at 37 °C for 5 h. Free GSNO was removed by the addition of 2 vol of −20 °C acetone and incubation at −20 °C for 1 h followed by centrifugation at 12,000*g* for 10 min at 4 °C; the supernatant was removed and this step was repeated three times. The rePON1-NO pellet was redissolved in HEN buffer and rePON1-NO levels were detected by Q-TOF LC/MS and Saville–Griess assay.

2.4. GSH addition to rePON1-NO

The resultant rePON1-NO/rePON1 mixture (62 μM in HEN buffer) was reincubated with or without (control) GSH (final concentration 2.5 mM) overnight at 25 °C. The amount of rePON1-NO was then analyzed by Q-TOF LC/MS.

2.5. Dose and time effect of GSNO on rePON1-NO level and stability

RePON1 (1.25 μM, 50 μg/mL) was dissolved in HEN buffer and incubated with 6.25, 12.5, 25 and 50 μM GSNO for 1 and 5 h at 37 °C. Excess GSNO was removed by filtration through an Amicon ultracentrifugal filter device (MW cutoff 10,000 Da; Millipore) and redissolved in PBS. The solutions were then analyzed by Q-TOF LC/MS immediately and after incubation at 4 and 37 °C for 1 week and 3 days, respectively, to determine levels of rePON1-NO and examine the stability of the SNO bond to rePON1 protein at these temperatures.

2.6. S-nitrosylation of rePON1 using HSA-NO

HSA (100 μM) was dissolved in PBS buffer, DTT (final concentration 1 mM) was added and the solution was incubated at 37 °C for 1 h. Excess DTT was removed by filtration through an Amicon ultracentrifugal filter device (MW cutoff 30,000 Da). The protein was then redissolved in HEN buffer. GSNO (1 mM) was added to the solution and incubated at 37 °C for 1 h in the dark. Excess GSNO was removed by filtration through the Amicon ultracentrifugal filter device (MW cut off 30,000 Da) and the solution was injected into the Q-TOF LC/MS to obtain 40% nitrosylation of HSA.

rePON1 (1.25 μM in HEN buffer) was incubated with 6.25, 12.5, 25 and 50 μM HSA-NO/HSA mixture for 1 and 5 h at 37 °C and nitrosylation level of rePON1 was determined by Q-TOF LC/MS.

2.7. Saville–Griess assay

Protein solution (20 μL) was added to 180 μL of solution A [1% sulfanilamide and 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride in 1% HCl solution) perwell of a 96-wellplate. Three repeats were performed for each protein solution. In a separate well, 20 μL protein solution was added to 180 μL solution B (solution A containing 1 mM $HgCl_2$), with three repeats for each protein solution.

After 30 min incubation at room temperature, the samples were measured spectrophotometrically at 540 nm. Amounts of rePON1-NO were determined by subtracting the absorbance of samples insolution A from the absorbance of samples insolution B. GSNO at concentrations ranging from 2.5 to 80 μM was used to create standard curves.

2.8. LC/MS analysis

Reverse-phase LC separation of rePON1/rePON1-NO was carried outina 1290 infinity LC system (Agilent Technologies). The protein was separated in a C-18 reverse-phase column (Zorbax 300 SB-C182, 1 × 50 mm 1.8 μm) with a 10-min gradient of solvent A [DDW with 0.1% formic acid (FA)] and solvent B (Acetonitrile-ACN and 0.1% FA) started at 0 min with 5% solvent B and raised to 90% solvent B at 5 min and 98% at 7 min at a flow rate of 0.2 mL/min. The LC eluent was directly introduced into the electrospray ionization (ESI) source connected to the Q-TOF MS.

2.9. Mass spectrometry

Protein analyses were carried out on an UHD accurate-mass Q-TOF LC/MS 6540 (Agilent Technologies). The ESI capillary voltage was set at 3500 V, fragmentor 150 V, gas temperature 300 °C, gas flow 9 mL/min and nebulizer 40 psig. The mass spectra (m/z 100–3000) were acquired in positive-ion mode. Deconvolution of the m/z spectra to the exact mass of the protein was generated using the maximum entropy algorithm with a mass range of 35,000.0–45,000.0 Da, mass step 1.0 Da, S/N threshold 50 and m/z range 800.0–2500.0.

2.10. Statistical analysis

Statistical analysis was carried out using the Excel 2010 program. Student's paired t-test was used to compare the means of two groups. Each experiment was repeated separately at least two or three times. Results are presented as mean ± SD.

3. Results

3.1. S-nitrosylation of rePON1 using GSNO

Our previous studies showed that oxidation of the free thiol of rePON1 at Cys-284 affects the enzyme's activity [18]. Here we examined PON1's ability to undergo S-nitrosylation at its free thiol. rePON1 (62 μM) was incubated with GSNO (25 mM) in HEPES buffer pH 7.4 containing metal-ion chelators (1 mM EDTA and 0.1 mM neocuproine) for 5 h at 37 °C. The protein was precipitated with acetone to remove excess GSNO and formed GSH. The protein precipitate was redissolved in HEPES buffer and injected into a Q-TOF LC/MS at a concentration of 1.25 μM to analyze S-nitrosylated protein level. The mass spectrum obtained for rePON1 (Fig. 1A) showed a Gaussian distribution of m/z peaks; upon incubation with GSNO, each peak split into two (Fig. 1B). Deconvolution of the m/z spectra using the maximum entropy algorithm gave a single peak with a mass of 40,415 for rePON1 (Fig. 1C) and two peaks

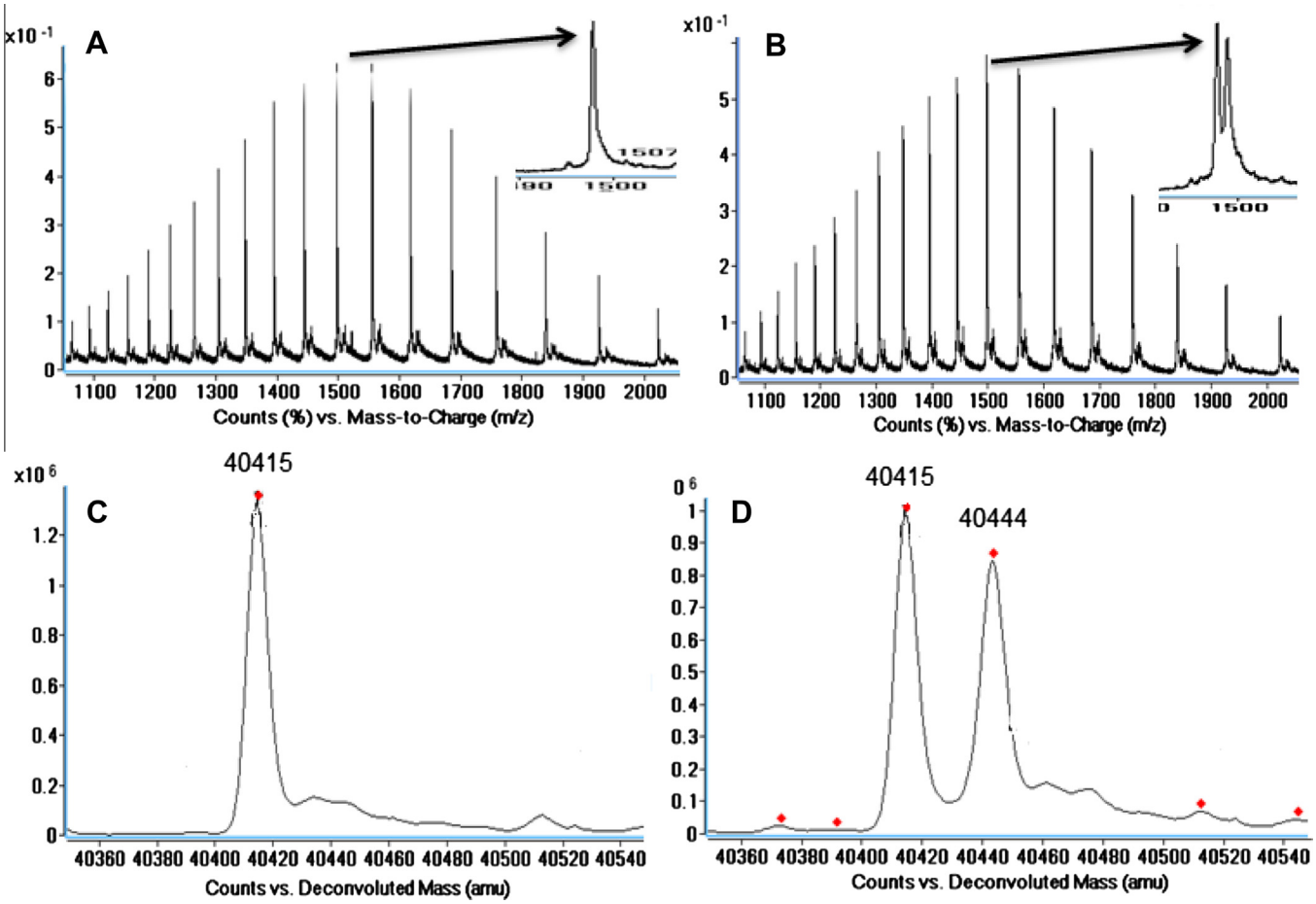

Fig. 1. LC/MS analysis of rePON1 and rePON1-NO. (A) MS analysis of rePON1 in Q-TOF MS (62 μM in 50 mM HEPES buffer pH 7 containing 1 mM EDTA and 0.1 mM neocuproine). (B) MS analysis of rePON1 after incubation with nitrosylated glutathione (GSNO, 2.5 mM) for 5 h. (C and D) Deconvoluted mass spectra of rePON1 (C) and rePON1 incubated with GSNO (D). The enzyme was injected into the Q-TOF at a concentration of 1.25 μM.

with masses of 40,415 and 40,444 for rePON1 incubated with GSNO (Fig. 1D); the 29 mass unit difference fit with the addition of NO to the protein. The ratio of the nitrosylated rePON1 calculated from the area obtained under each deconvoluted peak was 42 ± 6% (Fig. 2) of the total level of rePON1. Incubation of the nitrosylated rePON1 (rePON1-NO) (1.25 μM) with GSH

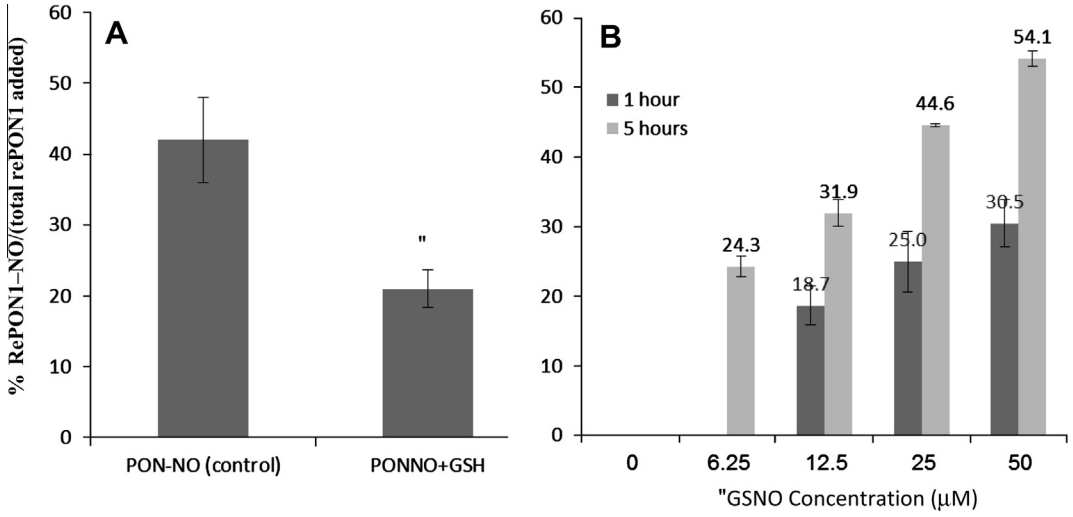

Fig. 2. (A) Glutathione (GSH) eliminates NO from rePON1-NO. Level of S-nitrosylated rePON1 (rePON1-NO) before and after addition of GSH. RePON1-NO/rePON1 mixture was incubated overnight at 25 °C with or without (control) 50 μM GSH and analyzed in MS. The experiment was repeated three times. (B) Level of rePON1-NO increases with increasing GSNO concentration. RePON1 (1.25 μM) was incubated with different concentrations of GSNO (6.25–50 μM) for 1 or 5 h at 37 °C and the percentage of rePON1-NO was analyzed by MS. Each experiment was repeated two times. Results are presented as mean ± SD.

(50 μM) overnight at 25 °C decreased the rePON1-NO/(total rePON1) ratio to 21 ± 2% (Fig. 2A). This suggests that the NO group bound to the enzyme's free thiol Cys-284 was transferred to the glutathione via trans-S-nitrosylation.

3.2. Analysis of rePON1-NO by Q-TOF LC/MS versus Saville–Griess assay

The Saville–Griess method is a colorimetric assay specific for determining protein S-nitrosylation. This method is based on the cleavage of NO from the protein-NO by the mercury ion Hg^{+2}; the released NO^{+} is trapped by sulfanilamide and *N*-(1-napthyl)ethylenediamine dihydrochloride mixture to form a chromophore that is strongly absorbed at 540 nm. Samples of rePON1-NO obtained by trans-S-nitrosylation with GSNO were analyzed by Q-TOF LC/MS and by Saville–Griess assay, and the results were compared (Table 1). The average of three separate experiments using Q-TOF LC/MS gave a rePON1-NO/(total rePON1) ratio of 42 ± 6% compared to 46 ± 11% obtained by the Saville–Griess assay (Table 1). These data provide further proof that PON1 undergoes S-nitrosylation upon incubation with GSNO.

3.3. rePON1 S-nitrosylation conditions and stability of rePON1-NO

The effect of GSNO concentration and incubation time on the rePON1-NO/(total rePON1) ratio was examined. RePON1 (1.25 μM) was incubated with different concentrations of GSNO (6.24–50 μM) for 1 or 5 h at 37 °C. The rePON1-NO/(total rePON1) ratio increased in a dose- and time-dependent manner. The S-nitrosylation of rePON1 was 30% when the protein was incubated with 40-fold excess GSNO for 1 h, whereas prolonging the incubation time to 5 h at 37 °C increased the rePON1-NO/(total rePON1) ratio to approximately 50% (Fig. 2B).

The stability of the NO moiety in the S-nitrosylated rePON1 was also tested. Excess GSNO was removed from the synthesized rePON1-NO and rePON1 mixture using a 10,000-Da-cutoff membrane and the resultant protein mixture was injected into the LC/MS immediately, and reinjected after storing the sample at 4 °C for 1 week or at 37 °C for 3 days (Fig. 3). There was a non-significant decrease in rePON1-NO level of about 10% after 1 week at 4 °C and a decrease of about 25% at 37 °C after three days. Thus S-nitrosylated rePON1 is relatively stable for 1 week at 4 °C and moderately stable for 3 days at 37 °C.

3.4. S-nitrosylation of rePON1 using HSA-NO

HSA is the most abundant protein in human serum and plasma. Similar to PON1 enzymes, HSA also contains one free cysteine, which can be S-nitrosylated by endothelial nitric oxide synthases (eNOS) and in fact, HSA could potentially be the first protein to be nitrosylated due to its high concentration. S-nitrosylated HSA (HSA-NO) has been suggested to act as a circulating reservoir for NO produced by endothelial cells [11]. The ability of HSA-NO to transfer its NO group to rePON1 via protein–protein trans-S-nitrosylation was thus examined.

Table 1
Quantitative analysis of rePON1-NO by Q-TOF LC/MS versus Saville–Griess assay.

Replicate	% of rePON-NO by LC/MS	% of rePON-NO by Griess	Deviation (%)
1	46.73	54.28	16.16
2	34.07	33.02	3.18
3	44.09	51.60	17.03
Average	41.9	46.3	10.5
STDEV	6.2	11.5	

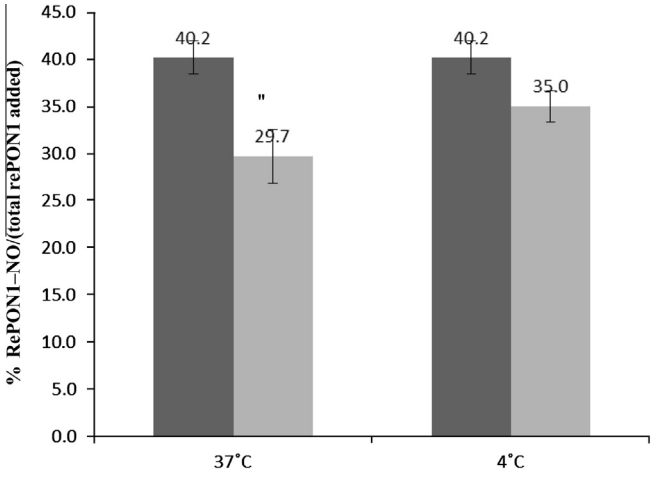

Fig. 3. Stability of rePON1-NO in buffer solution (PBS) at 4 °C for 1 week or at 37 °C for 3 days. RePON1 (1.25 μM) was incubated with GSNO (50 μM). GSNO was removed and the level of rePON1-NO was analyzed by MS immediately (black columns) and after 1 week at 4 °C or after 3 days at 37 °C (gray columns). Each experiment was repeated two times. Results are presented as mean ± SD.

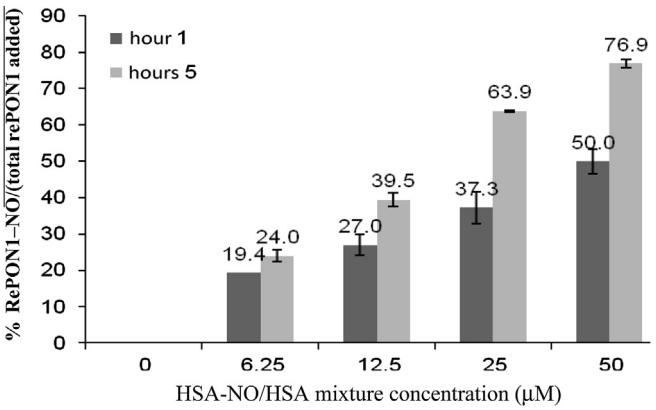

Fig. 4. Human serum albumin (HSA-NO) is very effective in trans-nitrosylation of rePON1. HSA was reduced with DTT to obtain free thiol and nitrosylated with GSNO to obtain 45% HSA-NO (MS method). RePON1 (1.25 μM) was then incubated with different concentrations of HSA-NO/HSA mixture (6.25–50 μM) for 1 and 5 h at 37 °C. The amount of S-nitrosylated rePON1 (rePON1-NO) was then analyzed by MS. Each experiment was repeated two times. Results are presented as mean ± SD.

To ensure that HSA cysteine was present in its free form, HSA was first reduced by incubation with DTT followed by the removal of excess DTT using a 30,000-Da-cutoff membrane. HSA was then nitrosylated with GSNO to obtain 43% HSA-NO (determined by LC/MS). After removal of excess GSNO using the 30,000-Da-cutoff membrane, rePON1 (1.25 μM) was incubated with different amounts of a HSA-NO/HSA mixture (6.25–50 μM) at 37 °C for 1 and 5 h and injected to Q-TOF LC/MS for analysis. The rePON1-NO/(total rePON1) ratio obtained after 1 h showed a dose-dependent increase from 19.5% with fivefold excess HSA-NO/HSA mixture to 50% with 40-fold excess, whereas after 5 h of incubation with 40-fold excess, rePON1-NO reached 76% (Fig. 4). This experiment revealed that in addition to GSNO, nitrosylated albumin can transfer its NO group to rePON1 via trans-S-nitrosylation, and this transfer was much more efficient than that by GSNO, even when the HSA is present at low excess versus PON1 relative to its excess in the serum. In fact, nitrosylation of rePON1 resulted in the highest rePON1-NO level achieved by any method used in this work [GSNO or Cys-NO, S-nitroso-N-acetyl-D,L-penicillamine (SNAP), N-acetylcysteine-NO (NAC-NO) or the hydrophobic NO donor diethylamine NONOate].

4. Discussion

Protein PTM plays an essential role in the biological regulation of the cell. PTMs are extremely important because they can change a protein's physical or chemical properties, conformation, activity, cellular location or stability, an ability that might be part of their evolutionary development [1,2,21].

PON1's activity is critical to HDL's antiatherosclerotic properties and we hypothesized that PON1's properties might be altered by specific modifications and by either physical or covalent binding to metabolites. Such modifications can alter the protein's 3D structure; they might occur in vivo and can potentially be identified in lesions or sera of atherosclerotic patients [13]. PON1 is an enzyme containing a free thiol group at Cys-284 that is susceptible to oxidation and may be a sensor of environmental changes, particularly oxido/redox alterations. In recent years, protein S-nitrosylation, the covalent modification of NO on cysteine thiol, has been recognized as a main mechanism by which NO modulates protein activity and transduces NO bioactivity, and as an important element in the PTMs that regulate protein function and cell signaling [10].

The ability of PON1 to undergo S-nitrosylation was examined using several R-SNO donors via trans-S-nitrosylation mechanisms, including Cys-NO, SNAP, NAC-NO, GSNO and the hydrophobic NO donor diethylamine NONOate. Among these NO donors, GSNO was the most effective with rePON1, and it was therefore used as the SNO donor throughout this study.

Several methods have been developed to detect S-nitrosylated proteins: colorimetric methods such as the Saville–Griess assay, NO-specific antibody to detect in-situ protein S-nitrosylation using immunohistochemistry assay, and the use of a biotin-switch method coupled with immunoblotting, 2D-gel electrophoresis or fluorescence gel electrophoresis to detect changes in protein S-nitrosylation status under different biological conditions [22–24]. However, some of these methods are indirect which requiers release of the NO by metal ions or ascorbate, and trapping of the released moiety to form specific chromophore or fluorophore compounds, and others require large amounts of protein.

MS can be used to analyze and identify S-nitrosylated protein directly, while minimizing the chance of inducing false-positives during sample preparation. Published reports have suggested that under soft ESI conditions, it is possible to detect S-nitrosylated peptides. S-nitrosylated proteins have been observed as the addition of a +29 Da ion (M−H + 30) to the unmodified protein mass due to cysteine nitrosylation [3,23,25]. In the present study, the S-nitrosylated rePON1 was analyzed using Q-TOF LC/MS. Gaussian m/z peaks were obtained for rePON1 which split after nitrosylation of the enzyme. Deconvolution of the m/z spectra using the maximum entropy algorithm generated the molecular mass of the enzyme (40,415 Da), with an additional peak of 40,444 Da, indicating the addition of NO to rePON1 (Fig. 1). The ratio between rePON1-NO and total rePON1 was also calculated, indicating 40% nitrosylation. Incubation of the nitrosylated rePON1 with GSH decreased the level of nitrosylated enzyme by 50%, indicating that the NO group is connected to the free thiol of rePON1 and is transferred to GSH by trans-S-nitrosylation (Fig. 2A). Levels of rePON1-NO analyzed by Q-TOF LC/MS were well correlated to those obtained by Saville–Griess method, providing additional evidence that the NO group is indeed connected to rePON1's free thiol.

Levels of rePON1-NO increased in a dose- and time-dependent manner when rePON1 was incubated with GSNO at 37 °C. Maximum nitrosylation (54% rePON1-NO) was obtained by incubation of rePON1 with 40-fold excess GSNO for 5 h at 37 °C (Fig. 2B).

R-SNO is a labile bond and its stability varies with protein type and the thiol group's milieu inside and outside the protein [23]. Our results show that the SNO bond in rePON1-NO is stable for at least 1 week at low temperature (4 °C), whereas at 37 °C, the SNO bond is less stable: after 3 days the level of rePON1-NO/(total rePON1) decreased by 25% (Fig. 3).

Recent studies have shown that protein–protein trans S-nitrosylation can occur where an NO moiety is transferred from one cysteine residue to another protein's cysteine residue, and this seems to be an important nitrosylation mechanism [26]. HSA-NO is suggested to act as a circulating reservoir for NO produced by endothelial cells and thus can serve as a NO donor to other proteins, such as PON1, circulating with HDL in the blood [27]. Examining this possibility, we found that nitorsylated HSA is a more effective NO donor to rePON1 than small molecules such as nitorsylated glutathione or cysteine. Incubation of rePON1 with 40% excess of a mixture of HSA-NO/HSA for 5 h at 37 °C resulted in 76% conversion of rePON1 to rePON1-NO, the highest amount of nitrosylated rePON1 achieved by any of the NO donors used in this study. HSA concentration in the serum is 0.6 mM, whereas that of PON1 bound to HDL is about 2.5 μM [28,29], suggesting that HSA is present at 200 times higher concentration than PON1 in serum. We found that the HSA-NO/HSA ratio following NO donation to rePON1 does not change much (data not shown), suggesting that NO transfer occurs via a direct interaction between the two proteins and not via the release of NO to the solution and then reaction of this species with rePON1. If the NO were transferred between the two proteins via the solution, the HSA-NO/HSA ratio would be expected to decay at a constant rate with time, independent of the presence and amount of rePON1 in the solution. Thus it seems that the two proteins interact directly and possess redox potentials that allow electron transfer, whereas protein apposition facilitates NO transfer between them.

In summary, as part of our effort to study the effect of PTMs on rePON1 activities and properties, we found that rePON1 can be S-nitrosylated and directly identified by Q-TOF LC/MS. GSNO and HSA-NO could be used for the trans-S-nitrosylation reaction, and the latter was found to be the most efficient NO donor to rePON1 in this study. Further research is required to elucidate the mechanism by which HSA-NO nitrosylates rePON1, and to determine PON1-NO's biological properties and its presence under normal and pathological conditions.

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