Peroxynitrite-mediated oxidative protein modifications

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Abstract Proteins are targets of reactive species and detection of oxidatively modified proteins is often used as an index of oxidative stress. Peroxynitrite is a strong oxidant formed by reaction of nitric oxide with superoxide. Using fatty acid-free bovine serum albumin as a model we examined peroxynitrite-mediated protein modifications. The reaction of protein with peroxynitrite resulted in the oxidation of tryptophan and cysteine, in the nitration of tyrosine, in the formation of dityrosine, in the production of 2,4 dinitrophenylhydrazine-reactive carbonyls and in protein fragmentation. The formation of 3-nitrotyrosine represents a specific peroxynitrite-mediated protein modification that is different from modifications mediated by reactive oxygen species.

Key words: Nitrotyrosine; Dityrosine; Protein carbonyl; Tryptophan oxidation; Protein fragmentation

1. Introduction

Peroxynitrite is formed by the reaction between two free radicals, nitric oxide and superoxide:

$$\cdot NO + O_2^- \rightarrow ONOO^-$$

Peroxynitrite is not a free radical because the unpaired electrons of nitric oxide and superoxide are combined to form a new bond in peroxynitrite [1]. The second order rate constant of the reaction between nitric oxide and superoxide is 6.7×10^9 $M^{-1} \cdot s^{-1}$ [2]. The rate of the nitric oxide reaction with superoxide is approximately thirty times faster than the reaction of nitric oxide with oxyhemoglobin and three times faster than the reaction of superoxide with superoxide dismutase. Stimulated macrophages, neutrophils and endothelial cells have been shown to generate peroxynitrite [3–5] and recent data has provided evidence for in vivo formation of peroxynitrite in human atherosclerotic coronary vessels, in human acute lung injury and in chronic inflammation [6–9].

Peroxynitrite or its reactive intermediates can be toxic by reacting with several biological molecules. Peroxynitrite will oxidize unsaturated fatty acids in the absence of a metal catalyst to form thiobarbituric acid reactive substances and conjugated dienes [10], and to oxidize low molecular mass antioxidant molecules; glutathione, ascorbate and α-tocopherol [11–13]. We have previously found that the spontaneous reaction of peroxynitrite with proteins results in the nitration of the ortho positions of tyrosine residues to form nitrotyrosine [14]. The peroxynitrite-mediated nitration of tyrosine residues can also be catalyzed by Fe^{3+}-EDTA and Cu,Zn superoxide dismutase [15]. Nitrotyrosine has been detected in human pathologies [6–9] and is a useful marker for peroxynitrite detection. Here we examined additional protein modifications induced by peroxynitrite. We found that peroxynitrite induced the formation of protein carbonyls, the oxidation of tryptophan, tyrosine and cysteine residues and to cause protein fragmentation.

2. Materials and methods

2.1. Exposure of proteins to peroxynitrite

Peroxynitrite was synthesized from nitrite and H_2O_2 as described previously [1]. The concentration of peroxynitrite was measured by the increase in absorbance at 302 nm ($\varepsilon_{302\ nm}$ = 1,670 $M^{-1} \cdot cm^{-1}$) in 1.2 N NaOH. Fatty acid free bovine serum albumin (BSA, Boehringer-Manheim, Indianapolis, IN) at concentration of 4 mg/ml was reacted with chemically synthesized peroxynitrite in 100 mM potassium phosphate buffer, 100 μM diethylenepentaacetic acid (DTPA), pH 7.4. The reaction was performed by placing a small aliquot of peroxynitrite (4–8 μl) in the side of a tube containing the BSA solution immediately followed by vigorous vortexing. To control for the potential effect of nitrite, nitrate and H_2O_2 which are present in peroxynitrite [1], peroxynitrite was allowed to decompose for 5 min in the phosphate buffer before the addition of BSA (= 'reverse order'). To insure that peroxynitrite was decomposed, we measured the absorbance of peroxynitrite in 100 mM potassium phosphate buffer, 100 μM DTPA, pH 7.4 at 302 nm. The absorbance at 302 nm of the decomposed peroxynitrite was the same as the buffer alone.

2.2. Lung perfusion

We utilized a blood-free isolated lung preparation [16]. All animal studies in this work have been approved by the Institutional Animal Care and Use Committee. Sprague–Dawley, adult, male rats (Charles River Breeding Laboratories, Kingston, NY) weighing 150–170 g were anesthetized and a tracheostomy was performed. The lungs were isolated and ventilated continuously with 95% oxygen/5% carbon dioxide throughout the duration of the experiments. The lungs were perfused at constant flow rate of 12 ml/min with a recirculating Krebs–Ringer bicarbonate buffer, pH = 7.4 containing 3% (w/v) fatty acid free-BSA and 10 mM glucose that was maintained at 37°C and equilibrated with the same gas mixture as the ventilation gas. Lungs were made ischemic by stopping the flow for 2 h while maintaining ventilation.

2.3. Analytical methods

After completion of the experiment the lungs were rapidly clamped frozen in liquid nitrogen. Lungs were homogenized under nitrogen in ice cold saline (1:10 tissue/medium) containing 0.01% butylated hydroxytoluene. 2,4 dinitrophenylhydrazine (DNPH)-reactive protein carbonyls in lung homogenates were analyzed as described previously [16]. To account for the presence of nitrotyrosine in the DNPH-reacted samples (DNPH and nitrotyrosine absorb maximally at 358 nm at acidic pH), all samples were divided to two aliquots that contained equal amount of protein. One aliquot was reacted with 0.05% (wt./vol.) DNPH (Aldrich, Milwaukee, WI) in 1 M HCl and the other with 1M HCl for one hour at 37°C. At the end of the incubation, the samples were passed through a Sephadex G-25 column using 50 mM phosphate buffer pH 6.0. The difference in absorbance at 358 nm (DNPH reacted-HCl reacted) was used to estimate the concentration of the DNPH-

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reactive protein carbonyls using $\varepsilon_{358\ nm} = 21{,}000\ M^{-1} \cdot cm^{-1}$. The yield of nitration was determined by measuring the absorbance at pH 11.5, ($\varepsilon_{430\ nm} = 4{,}400\ M^{-1} \cdot cm^{-1}$). Formation of dityrosine was monitored by the absorbance at 330 nm using the $\varepsilon_{330\ nm} = 4{,}000\ M^{-1} \cdot cm^{-1}$. Oxidation of tryptophan was followed by the changes in fluorescence (excitation $\lambda = 325$ nm and emission $\lambda = 410$ nm). The fatty acid free BSA samples were analyzed on 10% SDS gels and silver stained following the completion of the analysis. For amino acid analysis fatty acid free-BSA was reacted with peroxynitrite as described above and then hydrolyzed in vapor phase with 6 N HCl containing 1% phenol for 24 h at 110°C. The hydrolyzed samples were derivatized at room temperature with phenylisothiocyanate (PTC) and the derivitized amino acids were separated on a reverse phase Waters PicoTag column (3.9 × 150 mm). The PTC-amino acids in the hydrolysates were quantified by comparing their retention times and peak areas with a freshly derivitized standard mixture of amino acids containing authentic nitrotyrosine (Aldrich, Milwaukee, WI).

3. Results and discussion

Fatty acid free-BSA reacted with the same volume of different peroxynitrite concentrations in order to achieve an 10:1, 1:1, 1:5 and 1:10 ratio of protein to peroxynitrite. We used a solution of 60.6 μM BSA in 100 mM potassium phosphate buffer to control for changes in pH because the chemically synthesized peroxynitrite is stored in 1.2 M NaOH. Since peroxynitrite decomposes in phosphate buffer with a pseudo first order rate of 1.3 s^{-1} at 25°C and pH 7.4, a concentration of 606 μM peroxynitrite – to give a ratio of 1:10 – will correspond approximately to a steady state exposure of 8.5 μM for 1 min. Figure 1 shows that a molar ratio of 10:1 did not result in formation of protein carbonyls. However, reacting the protein at ratio of 1:1 or greater resulted in a concentration dependent increase of protein carbonyls. Similarly increasing the concentration of peroxynitrite to a molar ratio of 1:1, 1:5 and 1:10 resulted in the oxidation of 41, 74 and 77% ($n = 3$) of the single free cysteine residue, respectively. The oxidation of the cysteine residue by peroxynitrite to form a protein-thiyl radical has been recently reported [17].

The reaction of protein with peroxynitrite resulted in a decrease of tryptophan fluorescence (Fig. 2A). The maximal tryptophan fluorescence of the unreacted protein was taken to represent 121.2 μM because BSA contains two tryptophan residues. A 12, 29 and 45% oxidation of tryptophan residues was observed following the reaction of BSA with 1:1, 1:5 and 1:10 ratio of protein to peroxynitrite, respectively. The fluorescence

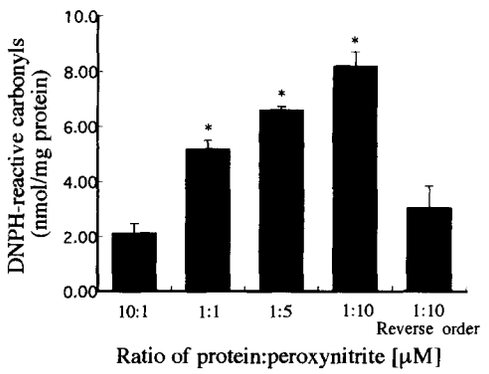

Fig. 1. DNPH-reactive carbonyl formation following treatment with peroxynitrite. Unreacted protein contained 2.1 ± 0.4 nmol/mg protein. The results represent 3 different preparation of fatty acid free-BSA and are expressed as mean ± S.D.*$P < 0.05$ compared to unreacted and reverse order of addition.

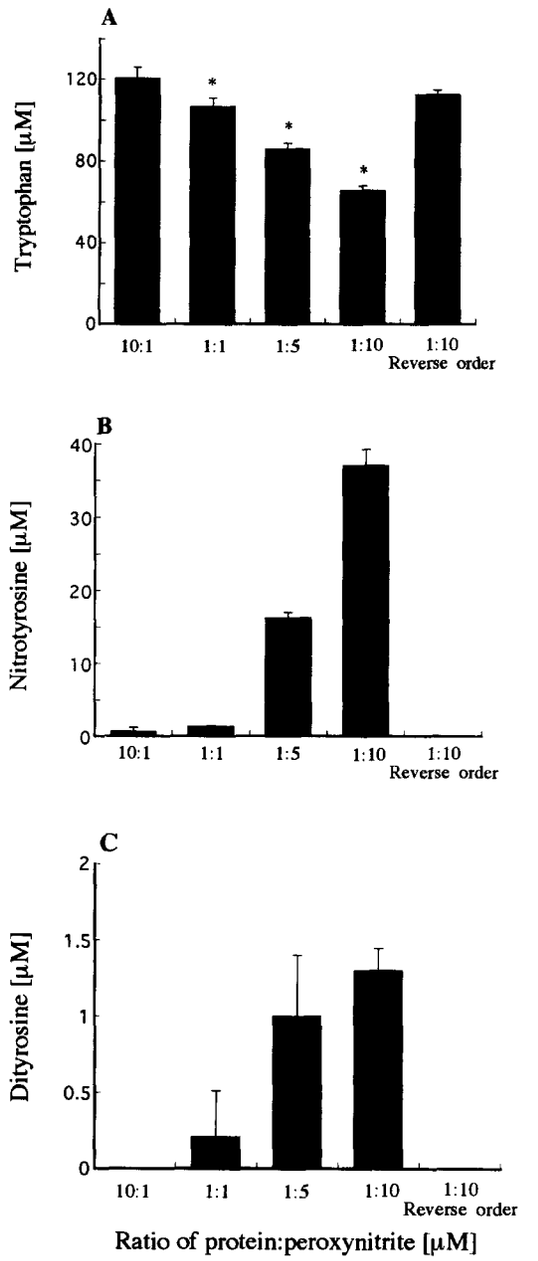

Fig. 2. Peroxynitrite-mediated modification of aromatic acid residues. (A) Loss of tryptophan fluorescence. (B) Formation of 3-nitrotyrosine. (C) Formation of dityrosine. Results represent 3–6 different preparation of fatty acid free-BSA and are expressed as mean ± S.D.

of tryptophan is dependent on the integrity of the indole ring. Disruption of the ring by oxidation or N-nitration may be responsible for the loss of fluorescence. If N-nitration of the indole ring occurs then this may be another specific modification mediated by peroxynitrite that will be useful for in vivo detection. BSA contains 19 tyrosine residues and 1:1, 1:5 and 1:10 ratio of protein to peroxynitrite 0.12, 1.4 and 3.2% of the tyrosine residues were nitrated (Fig. 2B). The nitration of tyrosine residues was also confirmed by amino acid analysis. Based on the absorbance at 430 nm at alkaline pH the yield of nitrotyrosine of the peroxynitrite-treated BSA samples at 1:10 ratio was 8.4 ± 0.5 μg/mg of protein which was the same as the

amount of nitrotyrosine detected by amino acid analysis 9.8 ± 1.8 μg/mg (n = 3) of protein. At 1:1, 1:5 and 1:10 ratio of protein to peroxynitrite 0.02, 0.09 and 0.11% of tyrosine residues were oxidized to dityrosine. The low yield of dityrosine formation is also confirmed by the absence of significant protein aggregation revealed by SDS-PAGE analysis (Fig. 3). However, the reaction of fatty acid free-BSA with peroxynitrite resulted in protein fragmentation as shown by the increase in band intensity of protein fragments with molecular weight lower than 66,000 (Figure 3). Furthermore, amino acid analysis of the protein reacted at ratio of 1:10 in addition to decrease in tyrosine residues also revealed a decreased recovery of more than 5%, as compared to control, of glutamic acid, histidine threonine and alanine (not shown). Histidine has been found to be sensitive to oxidation by reactive oxygen species as well as metal ion catalyzed oxidations [18]. Although not detected in this model system peroxynitrite has been shown to hydroxylate phenylalanine [19] and to oxidize methionine in α1-proteinase inhibitor [20].

Since the reaction of BSA with peroxynitrite resulted in the formation of DNPH-reactive protein carbonyls we examined the ability of peroxynitrite to form protein carbonyls in a preparation of lung homogenates. It was shown that ischemic injury to the lung results in an increase of protein carbonyls [16]. Lung

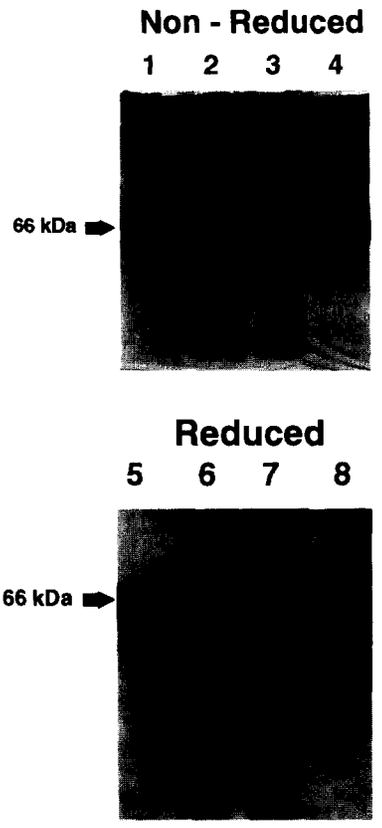

Fig. 3. Silver staining of SDS-PAGE analysis of peroxynitrite-treated samples. The same results were reproduced for at least 3 different protein preparations analyzing 20 μg of protein per lane. Lane: 1, reverse order of addition at 1:10 molar ratio of protein to peroxynitrite; 2, protein reacted at 1:10 molar ratio; 3, protein reacted at 1:5 molar ratio; 4, unreacted protein; 5, unreacted protein reduced with β-mercaptoethanol; 6, protein reacted at 1:5 molar ratio; 7, protein reacted at 1:10 molar ratio; and 8, reverse order of addition at 1:10 molar ratio of protein to peroxynitrite.

Table 1
DNPH-reactive protein carbonyls in lung homogenate

	DNPH-reactive protein carbonyls (nmol/mg of protein)
Control 2 h perfusion	2.2 ± 0.1
2 h Ischemia	6.9 ± 0.4*
10 mM tert-Butyl hydroperoxide + 100 μM Fe^{2+}	7.4 ± 0.5*
300 μM peroxynitrite	11.2 ± 0.7*,**

Isolated rat lungs were perfused or made ischemic as described in methods. Lung homogenate aliquots containing 4–5 mg protein/ml were reacted with 10 mM tert-Butyl hydroperoxide plus 100 μM Fe^{+2} for 30 min at 37°C and with peroxynitrite at room temperature. Data of three different lung homogenates is expressed as mean ± SD, *different than control, **different than 10 mM tert-butyl hydroperoxide plus 100 μM Fe^{2+}.

homogenates containing approximately 4 mg/ml protein were reacted with 300 μM peroxynitrite. This treatment resulted in the formation of 11.2 ± 0.7 nmol/mg of protein DNPH-reactive carbonyls that was significantly higher than control and than the levels of carbonyls formed after treatment of the same lung homogenates with 10 mM tert-butyl hydroperoxide in the presence of 100 μM ferrous iron for 30 min at 37°C (Table 1). The levels of DNPH-reactive carbonyls formed by 300 μM peroxynitrite which corresponds to an exposure of 4 mM for 1 min were also higher than the levels measured after 2 h of ischemia suggesting that peroxynitrite can contribute to in vivo protein carbonyl formation. Furthermore, we have recently found that the levels of protein carbonyls in ischemic rat lungs are significantly reduced by blockade of nitric oxide production [21]. Overall, peroxynitrite induced an array of protein modifications that arranged in order of magnitude include; cysteine oxidation, tryptophan oxidation, tyrosine nitration, formation of protein carbonyls, formation of dityrosine and protein fragmentation. The nitration of tyrosine residues and possibly of tryptophan are useful markers for detecting peroxynitrite in biological samples. Although the protein induced modifications of BSA by peroxynitrite can be considered to contribute to the antioxidant action of albumin in extracellular fluids [17], nitration of tyrosine residues and other peroxynitrite-mediated modifications inactivate important proteins such as Mn superoxide dismutase [14], surfactant protein A [22] and α-1 proteinase inhibitor [20] and may contribute to toxicity in human disorders.

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