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Effects of peroxynitrite-induced protein modifications on tyrosine phosphorylation and degradation

Andrew J. Gow[a], Daniel Duran[a], Stuart Malcolm[c], Harry Ischiropoulos[a,b,*]

[a]*Institute for Environmental Medicine, University of Pennsylvania, School of Medicine, 1 John Morgan Building, 3620 Hamilton Walk, Philadelphia, PA 19104-6068, USA*
[b]*Department of Biochemistry and Biophysics, University of Pennsylvania, School of Medicine Philadelphia, Philadelphia, PA 19104, USA*
[c]*Biokinetics Research Laboratory, Temple University, Philadelphia, PA 19104, USA*

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Abstract The ability of protein tyrosine kinases to phosphorylate a synthetic peptide was inhibited 51% by peroxynitrite-mediated nitration of tyrosine. Exposure of endothelial cells to peroxynitrite decreased the intensity of tyrosine phosphorylated proteins and increased the intensity of nitrotyrosine-containing proteins. Peroxynitrite-modified BSA was degraded by human red blood cell lysates. However, human plasma in a concentration-, time-, and temperature-dependent manner, removed the protein nitrotyrosine epitope. These results suggest that tyrosine nitration interferes with phosphorylation and targets proteins for degradation. Specific enzymatic process(es) for removing nitrotyrosine may be present in vivo.

Key words: Nitrotyrosine; Phosphotyrosine; Proteolytic degradation; Nitric oxide; Superoxide

1. Introduction

Peroxynitrite, the product of the near diffusion limited reaction between nitric oxide (•NO) and superoxide, induces a series of oxidative protein modifications [1-5]. The nitration of the *ortho* position of tyrosine residues in proteins by peroxynitrite has been considered a specific marker for the detection of peroxynitrite in vivo [1,5]. As such, using a variety of methods for detecting nitrotyrosine, several reports have shown that nitrotyrosine is associated with the induction of oxidative stress in human and animal models of disease [6-19]. Although the above body of published data has suggested that nitrotyrosine represents a protein modification specific for peroxynitrite formation in vivo, the possible role of tyrosine nitration in cellular function has not been fully explored. For example, tyrosine phosphorylation is an important regulator of signal transduction in cells and nitrotyrosine formation may interfere with normal signal transduction pathways. Preliminary data by Kong et al. showed that peroxynitrite-mediated nitration of a single tyrosine residue in the cell cycle kinase cdc2 prevents tryrosine phosphorylation [20]. Data presented here suggests that nitrotyrosine formation is responsible for the inhibition of tyrosine phosphorylation in endothelial cells exposed to low levels of peroxynitrite.

Nitration of tyrosine residues has been utilized as a protein modification to study the role of specific tyrosine residues in the function of proteins. Chemically induced tyrosine nitration has been shown to inactivate nearly 140 mammalian proteins whose activity is dependent on tyrosine residues [21]. Thus far peroxynitrite-mediated nitration of tyrosine residues has been shown to inactivate the mitochondrial Mn superoxide dismutase [1], the lipid aggregatory activity of surfactant protein A [22], and glutamine synthetase activity [23]. In fixed and freshly prepared tissues nitrotyrosine is stable and can be easily detected by a variety of techniques including specific affinity-purified polyclonal and monoclonal antibodies, amino acid analysis, HPLC analysis and GC/MS. However, we have observed that the levels of nitrotyrosine in non-fixed tissue and plasma samples stored even at −80°C decline over time. Therefore, we examined the possibility that proteolytic and other degradative pathways may exist in these samples that were responsible for the loss of nitrotyrosine.

2. Materials and methods

2.1. Cell culture, methods for exposing cells to •NO, and $ONOO^-$ and Western blot analysis

Starter cultures of bovine pulmonary artery endothelial cells (BPAEC) were originally a gift of Dr. E.J. Macarack (Connective Tissue Research Institute, University of Pennsylvania). The cells were subcultured as necessary on 35 cm^2 plastic tissue culture plates. Studies were performed at 1–3 days postconfluence and at passage 3–6. For experimental use, confluent monolayers of BPAEC ($0.8–1.0\times10^6$ cells per well of a 24 cm^2 plate) were washed three times with Earle's balanced salt solution (supplemented with 2.5 mM HEPES and 10 mM glucose) and equilibrated at 37°C. The nitric oxide donor utilized in these studies was spermine-NO (Cayman Chemical Co., Ann Arbor, MI), •NO was released at 2 μmol/min. For exposure to $ONOO^-$, 3-morpholinosydnonimine (SIN-1) was utilized as described previously [24], to release $ONOO^-$ at 2 μmol/min for 2 h. Protein concentration in cell lysates prepared after sonication of harvested cells (10 s×3 times at 75 W) was determined by the Bio-Rad protein assay kit. Endothelial cell lysates were separated using 12% SDS-PAGE electrophoresis and transferred overnight to nitrocellulose. After blocking with 5% dry milk in Tris-buffered saline (TBS), the nitrocellulose was exposed to the anti-nitrotyrosine antibody at a concentration of 0.5 μg/ml or to monoclonal anti-phosphotyrosine antibody (Oncogene Science) in 0.5% dry milk in TBS for 1 h. The antibody binding was visualized by luminol-enhanced chemiluminescence (Amersham, Arlington Heights, IL).

2.2. Phosphorylation assay

The activity of the tyrosine kinases c-src and v-abl in phosphorylating nitrated and non-nitrated substrate was assayed using a protein tyrosine kinase assay as per manufacturer's instructions (Oncogene Science). Briefly, a modified gastrin analog (Raytide), containing a single tyrosyl residue, is used as a substrate. 0.1 units of enzyme (1 unit of enzyme catalyzes the incorporation of 1 pmol of phosphate per min from ATP onto tyrosyl residues) are reacted with 4 nmol of Raytide in 50 mM HEPES pH 7.5 containing 0.1 mM EDTA and 0.015% Brij 35. The reaction is started by adding 0.05 mM ATP containing 10 mM $MgCl_2$ and 2 μCi of [γ-^{32}P]ATP. The reaction mixture was incubated for 30 min at 30°C. Reaction was stopped by adding 10% phosphoric acid and then the reaction mixture was applied to p81 Whatman paper. The papers were washed with 0.5% phosphoric acid and acetone, dried, and quantited by liguid scintilla-

*Corresponding author. Fax: (1) (215) 898-0868.

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tion counting. To measure the phosphorylation of nitrated substrate, the Raytide was reacted with a 10-fold excess of chemically synthesized peroxynitrite.

2.3. Protein degradation assays

Fatty acid-free bovine serum albumin was labeled with ^{14}C by reductive methylation as described previously [25,26]. The protein was reacted with peroxynitrite in 100 mM potassium phosphate, 0.9% NaCl buffer that contained 100 μM of the metal chelator diethylenetriaminepentaacetic acid (DTPA), pH 7.4 to give a molar ratio of protein to peroxynitrite of 1:10. The yield of nitration was determined by measuring the absorbance at pH 11.5 (ε_{430nm} = 4400 M^{-1} cm^{-1}). Fresh plasma and red blood cells were collected from adult volunteers. The plasma was centrifuged at 10000×*g* and diluted with 100 mM potassium phosphate buffer, pH 7.4, to obtain aliquots with 1 mg protein/ml. Red blood cell lysates were prepared as described previously [25]. For proteolytic degradation 5 mg /ml of red blood cell lysate was incubated with 0.3 mg/ml of labelled nitrated protein and the proteolytic degradation was measured as described previously [25,26].

2.4. Nitrotyrosine degradation assay

A solid-phase immunoradiochemical assay was used to measure nitrotyrosine degradation. Tissue proteins, plasma and peroxynitrite-modified BSA were immobilized onto nitrocellulose using the 96-well Bio-Dot microfiltration unit (BioRad, Hercules, CA). This assay was developed previously and the binding characteristics of the antibody established [27]. Eight to twelve different concentrations of the peroxynitrite-modified BSA standard were loaded in duplicate for each blot. Four to eight concentration-dependent dilutions of the samples were loaded in 400 μl TBS. After blocking with 5% dry milk the nitrocellulose was incubated with the anti-nitrotyrosine antibody for 15 h followed by a 3 h incubation in solution containing a donkey anti-rabbit ^{125}I-labeled IgG (0.1–0.2 mCi/ml). The blot was extensively washed in Tween-TBS and dried. The radioactivity of each sample was measured directly by beta scanning using an Ambis 400 imaging detector. The net counts of radioactivity (corrected for background counts from a sample blank) were obtained using the Ambis image analysis software v4.1 and then plotted on a semi-logarithmic plot. The binding of the antibody was saturable and dependent on the concentration of the antibody and antigen. At antibody concentration of 2 μg/ml the binding of nitrotyrosine increased linearly with increasing antigen concentration up to 1 μg of nitrotyrosine. The linear portion of the antibody binding curve to immobilized antigen was utilized to study the effects of nitrotyrosine degradation.

3. Results and discussion

The hypothesis that nitrotyrosine formation in proteins interferes with tyrosine phosphorylation was tested: (1) In vitro using a synthetic commercially available peptide that is a modified gastrin analog with one tyrosyl residue at the carboxyl terminal. This peptide is a substrate for the tyrosine kinases *v-abl* ($p43^{v-abl}$) and *c-src* ($p60^{c-src}$). These tyrosine kinases are known to phosphorylate a number of cellular proteins after stimulation with growth factors and cytokines [28]. (2) In endothelial cell lysates after exposure of cells to peroxynitrite.

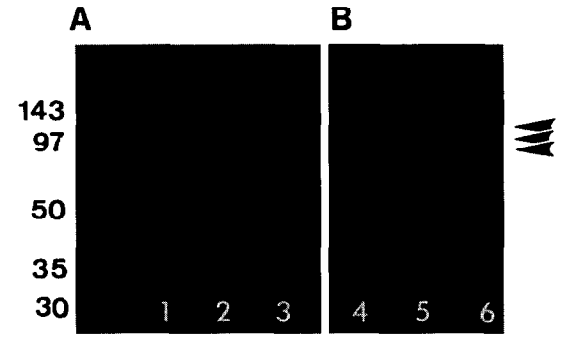

Fig. 1. Effects of peroxynitrite in tyrosine phosphorylation in bovine pulmonary artery endothelial cells. Equal amounts of endothelial cell lysate protein (20 μg) were separated on a 12% SDS-PAGE gel, transferred to nitrocellulose and probed with a monoclonal anti-phosphotyrosine antibody (A) and with an affinity-purified polyclonal anti-nitrotyrosine antibody (B). The data is a representative analysis of a duplicate experiment with the same tyrosine nitrated and phosphorylated bands found in the second analysis. Lanes: 1,6, control; 2,5, SIN-1 treated; 3,4, spermine-NO treated.

The phosphorylation of the 2.2 kDa synthetic peptide by purified tyrosine kinases ($p43^{v-abl}$ and $p60^{c-src}$) was inhibited by 51% (2037 ± 190 vs. 1007 ± 152 cpm, n = 3) when the peptide was first reacted with $ONOO^-$ to form nitrotyrosine. The peptide was reacted with peroxynitrite at a ratio of 1 to 10, which has previously been shown to yield 100% nitration of tyrosine residues [1]. It is important to note that the phosphorylation reaction is performed in a large excess of substrate (4 nmol of peptide to 0.1 units of enzyme). Therefore, this assay measures the effect of nitration on the rate of phosphorylation of the substrate. This reduced phosphorylation rate is consistent with the results reported by Martin et al.

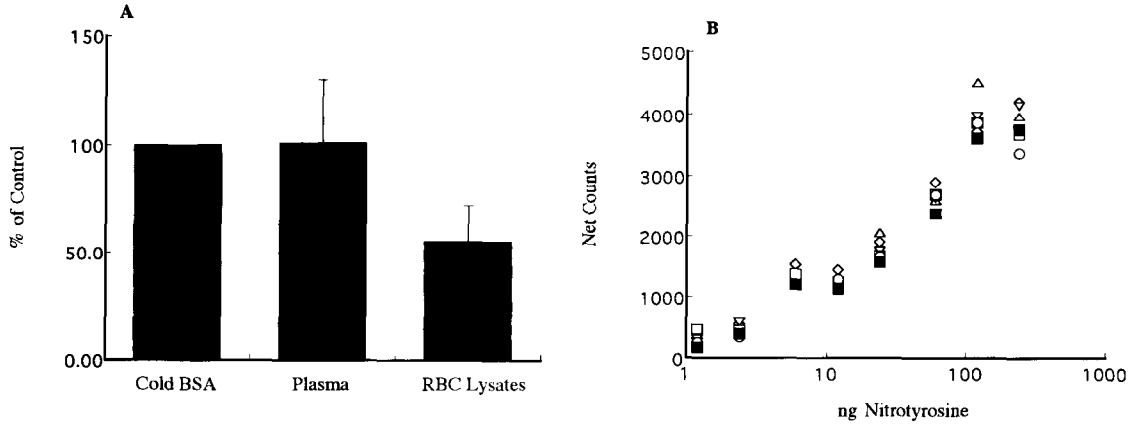

Fig. 2. (A) SDS-PAGE analysis of ^{14}C-labelled fatty acid-free BSA reacted with peroxynitrite at ratio of 1:10 (protein to $ONOO^-$) and incubated at 37°C for 60 min with cold BSA, human plasma and RBC lysates. Gels were subjected to scanning by Ambis 4000 and the intensity of the bands measured. 100% was set as the intensity of the band from the sample incubated with cold BSA. (B) Nitrated BSA was mixed with different concentrations of human plasma and blotted without incubation onto nitrocellulose.

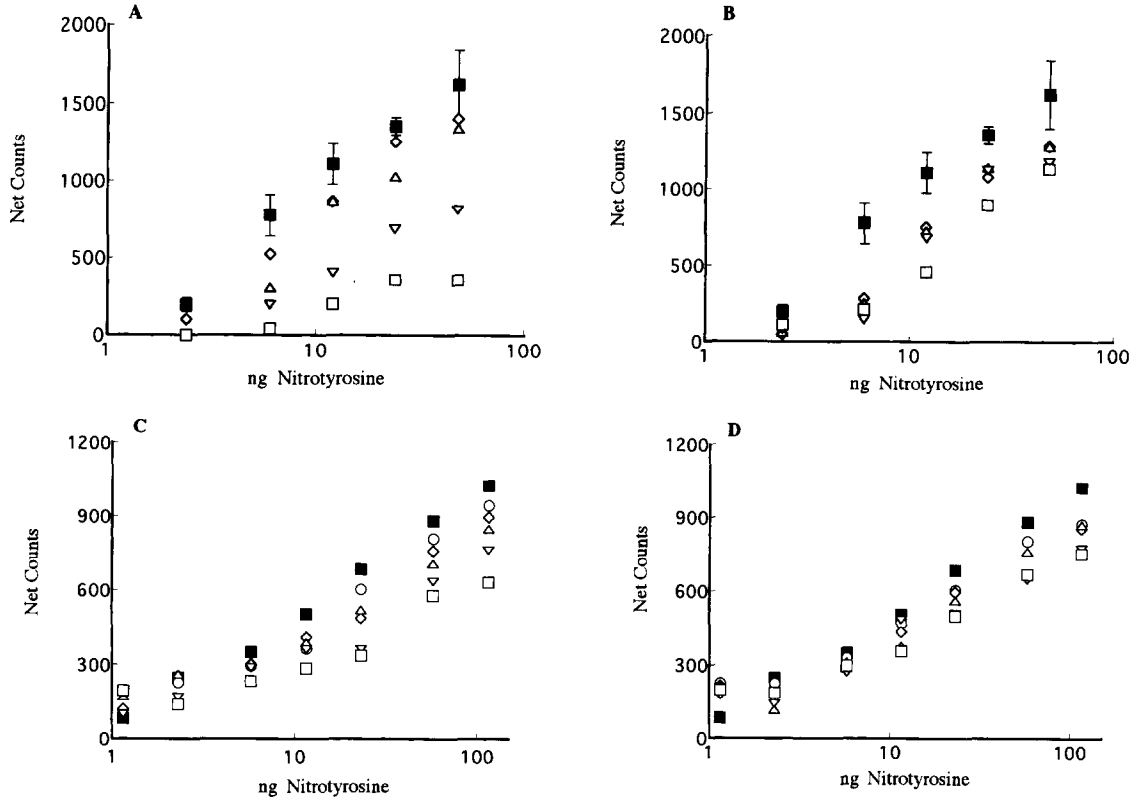

Fig. 3. (A) Concentration-dependent loss of antibody binding of nitrated BSA. Various concentrations of nitrated BSA were incubated with different protein concentrations of human plasma to give the following ratios; (■) 1:0, (◇) 1:0.005, (△) 1:0.05, (▽) 1: 0.5 and (□) 1:5 for 30 min at 37°C. (B) Same experiment as in A but the incubation was performed at 4°C. (C) Nitrated BSA was incubated with human plasma at 1:1 protein ratio and incubated for various time at 37°C. The control (■) is without plasma at 30 min and (○) indicates time zero, (◇) 5 min, (△) 10 min, (▽) 15 min and (□) 30 min. (D) Same experiment as in C but the incubation was performed at 4°C.

showing that *N*-acetylimidazole-mediated acetylation of the phenolic hydroxyl group in tyrosine is reduced when the *ortho* position of tyrosine is nitrated [29]. In addition, the same work showed that the phosphorylation of tyrosine is significantly affected by the pK_a of the the phenolic hydroxyl group. The normal pK_a of the hydroxyl group is 10.07 and substitutions that shifted toward lower pK_a values decreased the rate of phosphorylation [22]. Nitration of the *ortho* position of tyrosine shifts the pK_a of the aromatic hydroxyl group to 7.5 which may provide the mechanism for the significantly reduced rate of phosphorylation.

Confluent monolayers of BPAEC were exposed to either peroxynitrite or nitric oxide at 10 μM/min for 2 h. After exposure, cell lysates were prepared in the presence of protein phosphatase inhibitors (20 μM orthovanadate) and protease inhibitors (5 mM PMSF, 0.1 μg/ml aprotinin and leupeptin), and the cell lysate proteins were separated by 12% SDS-PAGE under reducing conditions (Fig. 1). Western blot analysis of endothelial cell lysates using monoclonal anti-phosphotyrosine antibodies revealed approx. 7 phosphorylated bands ranging from 50 to 150 kDa. The protein bands located approximately at 143, 100, 90, 80 and 70 kDa showed the greatest binding to anti-phosphotyrosine antibodies. The antibody binding of these bands was markedly reduced following exposure to $ONOO^-$ but to a much lesser degree following nitric oxide exposure (Fig. 1A). Analysis of endothelial cell lysates using an affinity-purified polyclonal anti-nitrotyrosine antibody demonstrated increased nitration of some of the protein bands which showed reduced binding with the phosphotyrosine antibody after $ONOO^-$ exposure (indicated by the arrows in Fig. 1B). The presence of nitrated proteins within control lysates is not surprising as nitrotyrosine has been previously detected in both control cell lysates and in normal human plasma [19]. These results suggest that $ONOO^-$ reduced tyrosine phosphorylation by nitrating tyrosine residue(s). Published data has shown that the activity of protein kinases and protein tyrosine phosphatases is reduced by exposure to oxidants, via oxidation of critical cysteine residues [30,31]. However, to establish the involvement of such a mechanism in this system requires further experimentation.

Davies and co-workers have provided extensive evidence that oxidative modification of proteins increases their susceptibility to proteolytic degradation [25,26]. Removal of modified proteins and increased protein turnover can be considered another line of defense against oxidative stress. Therefore, we tested if $ONOO^-$-reacted BSA was cleaved more efficiently than unreacted BSA by proteolytic enzymes present in human red blood cell lysates. Proteolytic degradation was measured by the percentage of ^{14}C that was acid soluble post incubation. After incubation of control BSA with red blood cell lysates $0.65 \pm 0.18\%$ ($n = 4$) of counts were acid soluble. $ONOO^-$-modified BSA was degraded more rapidly than the control, $4.95 \pm 0.90\%$ ($n = 4$) of counts being acid soluble. This increased degradation is confirmed by Fig. 2A, which shows a 50% reduction in labelled nitrated BSA post incubation with red blood cell lysates. Although peroxynitrite can modify other residues in BSA [5] under these experimental conditions, it appears that tyrosine nitration may also be a

signal for proteolytic degradation. Recently, Bartlett et al. showed that tyrosine nitration increases proteolytic degradation of glutamine synthetase [23].

Previously, while developing a solid-phase immunoassay for nitrotyrosine quantification in proteins, we discovered that human plasma and rat tissue homogenates from brain, lung and liver, had the ability to remove the nitrotyrosine epitope. Upon examination of the activity within plasma, it did not appear to be due to proteolytic degradation. SDS-PAGE analysis of peroxynitrite-modified fatty acid free BSA after incubation with plasma did not reveal loss of the ^{14}C label (Fig. 2A). For the solid-phase immunoassay, various dilutions of BSA that contained a known amount of nitrotyrosine were placed on nitrocellulose paper. After proper blocking, anti-nitrotyrosine antibody is allowed to react and the amount of the primary antibody binding was quantified by radioactivity counting on an Ambis scanner after addition of ^{125}I-labeled secondary antibody. As shown in Figs. 2 and 3, plots of log of concentration versus the net counts showed a linear relationship for concentrations of nitrotyrosine ranging from 5 to 100 ng. Plasma appears to remove nitrotyrosine in a time, concentration- and temperature-dependent manner (Fig. 3). This action resulted in the detection of less nitrotyrosine, as evident by the parallel shift to the right in the plots of log of concentration versus the net counts. Plasma added to nitrotyrosine-containing BSA immediately prior to addition of the primary antibody did not interfere with the ability of the antibody to detect nitrotyrosine (Fig. 2B). The loss of nitrotyrosine after incubation with plasma was confirmed by amino acid analysis. The nitrotyrosine content of $ONOO^-$-modified fatty acid free BSA was 0.15 ± 0.03 mol% ($n = 3$), whereas after treatment with human plasma, for 1 h at 37°C, content was reduced to 0.09 ± 0.01 mol% ($n = 3$). These observations raised an interesting question. How can we detect nitrated proteins in plasma if plasma degrades nitrotyrosine-containing proteins? We repeated the same experiment as in Fig. 3 (37°C and 30 min incubation), but instead of fresh plasma we used plasma that was reacted with $ONOO^-$. The plasma reacted first with $ONOO^-$ did not have nitrotyrosine-degrading activity, suggesting that the nitrotyrosine-degrading activity was inhibited by peroxynitrite. These results indicate that plasma and tissues can degrade peroxynitrite-modified proteins. Therefore, caution must be taken during sample analysis for nitrotyrosine. Inclusion of protease inhibitors and decrease time of storage may be necessary for succesful detection.

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