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N-NITROSATIONS OF BASIC AMINO ACID RESIDUES IN POLYPEPTIDE

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1. ABSTRACT

Changes in the electrophoretic pattern were noted in the products of polypeptides of identical basic amino acids preincubated with reactive or degraded PN, suggesting the occurrence of N-nitrosation of the ε-amino group of lysine, the guanido group of arginine and the imidazole group of histidine. Additionally, increase in the N-nitroso immunoreactivity of preincubated histones H2A and H2B was detected by Western blot analysis.

2. INTRODUCTION

Under certain physiological conditions, nitric oxide (NO) can react with superoxide free radicals and convert into peroxynitrite (PN) (1-3), a potent oxidative/nitrosative agent. Subsequently, PN can modify peptides/proteins in different ways. In addition to its tyrosine-nitration (1, 4-9) and S-nitrosation of thiols (1, 4, 5, 9-12), N-nitrosation of side chains (9, 12, 13) may also occur. The first indirect evidence of N-nitrosation was extracted via the observation that preincubation of poly basic amino acids with PN resulted in the significant alteration of UV spectra (12). Additionally, both N-nitrosation and denitrosation were suggested via the following findings: (i) increased immunoreactivity of ε-nitrosolysine in PN-preincubated histones H2A, H3 and H4; and (ii) decreased immunoreactivity in either subsequently dialyzed and stored or GSH- incubated histones (13). Thus far, the research of N-nitrosation is still in infancy because the detailed reaction-mechanism and the related biological significance are yet to be established. We, therefore, investigate whether the N-nitrosation of basic amino acids could lead to changes of electrophoretic patterns.

3. MATERIALS & METHODS

Poly-$_L$-Lysine (1.88 ~ 3.8 kDa and 68.6 ~ 75.9 kDa), Poly-$_L$-Arginine (7.5 ~ 14 kDa), Poly-$_L$-Histidine (39.2 kDa), sodium dodecyl sulfate (SDS), EDTA and anti-rat IgG-alkaline phosphatase (AP) conjugate were supplied by Sigma Chemical Company, St. Louis, MO, USA. Histones H1, H2A, H2B, H3 and H4 were purchased from Roche Applied Science, Indianapolis, IN, USA. Sodium nitrite was obtained from Fisher Scientific Company, Fair Lawn, NJ, USA. Reactive peroxynitrite (PN) and degraded PN were from Upstate Charlottesville, Virginia, USA. Anti-nitrosolysine and anti-nitrosohistidine were purchased from Calbiochem-Novabiochem Corporation, San Diego, CA, USA. Western blotting reagents nitrocellulose membranes, anti-rabbit IgG-alkaline phosphatase conjugate and Coomassie Blue G-250 stain and Silver stain were provided by Bio-Rad Laboratories, Hercules, CA, USA.

3.1. Nitrosation of poly amino acids and histones

The pre-incubation mixture was in a final volume of 30μl of 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA, 1 mM $FeCl_3^+$, 70~210 μm PN and approximately 30 μg of polypeptide. The reactions were carried out at 25°C for 15 minutes.

3.2. Analysis by SDS-PAGE and Western blotting

The above pre-incubated samples were subject to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (12%) at 4°C. The addition of reducing agent and the step of boiling were omitted in the sample treatment (5, 7, 9) to prevent the degradation of the nitroso group in SDS-PAGE. Coomassie brilliant blue G-250 and silver staining were employed to detect the intensity and/or mobility changes of modified polypeptide in the gels.

Additionally, Western blotting was performed to evaluate the sensitivity and specificity of commercial rabbit anti-nitrosolysine or rat anti-nitrosohistidine (0.2μg/ml) after SDS-PAGE. The step of transferring required pre-cooled tank buffer and prefrozen Towbin transfer buffer (5, 11). Anti-rabbit IgG-AP conjugate or anti-rat IgG-AP conjugate (0.1 μg/ml) was used as the secondary antibody.

4. RESULTS AND DISCUSSION

In this study, discrete defined bands were not observed because the polypeptide used was not homogenous and contained a wide range of molecular

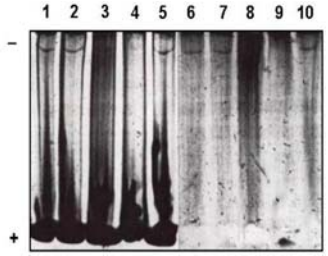

Figure 1. Modification of poly-$_L$-lysine analyzed by SDS-PAGE with silver staining. Polylysine samples used in lanes 1 to 5, and lanes 6 to 10 were with the molecular mass 1.8 ~3.8 and 68.8~75.9 kDa, respectively. Lanes 1 and 6, controls; lanes 2 and 7, controls with the addition of 70 μM degraded PN (after preincubation and prior to SDS-PAGE); lanes 3 and 8, preincubated with degraded PN (70 mM); lanes 4 and 9, preincubated with reactive PN (70 μM); and lanes 5 and 10, preincubated with $NaNO_2$ (10 mM).

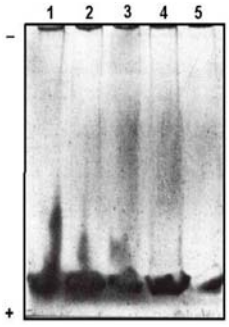

Figure 2. Modification of polylysine (1.8~3.8 kDa) analyzed by SDS-PAGE with Coomassie blue G-250 staining. Lane 1, control; lane 2, control with addition of 70 μM degraded PN (after preincubation) prior to SDS-PAGE; lane 3, preincubated with degraded PN (10 mM); lane 4, preincubated with reactive PN (70 μM); and lane 5, preincubated with $NaNO_2$ (10 mM).

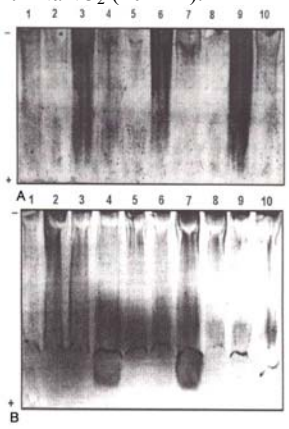

Figure 3. Modification of poly-$_L$-arginine analyzed by SDS-PAGE with: **A.** Silver staining and **B.** Coomassie blue G-250 staining. Lane 1, control; lanes 2, 5 and 8, controls with addition of degraded P.N, 70, 140 and 210 μM respectively (after preincubation and prior to SDS-PAGE) lanes; 3, 6 and 9, preincubated with degraded PN 70, 140 and 210 μM respectively; lanes 4, 7 and 10, preincubated with reactive PN, 70, 140 and 210 μM respectively.

mass. Apparently, there is an advantage in using a polypeptide made of an identical basic amino acid to determine a specific chemical modification (eg. nitrosation) because they contain numerous ε-amino, guanido, or imidazole groups (12). Modifications of these groups could be more conclusively detected because it is highly unlikely that modification would occur at the limited number of carboxyl and amino terminals (12). Possible nitrosations of polypeptides were discussed in previous studies in which the preincubation of polypeptides with PN/nitrite led to the alteration of UV absorption spectra (12). Moreover, the N-nitrosation of PN-reacted histones H2A, H3 and H4 was suggested via the increased immunoactivity of nitrosolysine (13). Conventional SDS-PAGE (12%) was found to be reliable in comparison with commercially precasted tricine or gradient gels, conventional SDS-PAGE (12%) was most suitable for our study. Silver staining revealed a change in mobility and intensity in high and low molecular mass of modified polylysine (Figure 1, lanes 3 and 8). Due to poor sensitivity of Coomassie blue R-250, G-250 was selected for more sensitive staining throughout the entire experiment. As a result, the polylysine in the range of low molecular mass was easily detected (Figure 2, lanes 3, 4 and 5). Nevertheless, because of the difference in the affinity/sensitivity between these two stainings, difference in mobility and intensity was found (Figure 1 and 2). The findings suggest that modification probably occurred at multiple free ε-amino groups.

The (reactive PN/degraded PN concentration)-dependent alteration in electrophoretic pattern of preincubated polyarginine was detected in both silver (Figure 3A) and Coomassie blue (Figure 3B) stained gels. A progressive increase in peptide-band intensity was noted as the concentration of degraded PN increased from 70μM up to 210μM (Figure 3A, lanes 3, 6 and 9, respectively). Inversely, the progressive decrease in intensity was observed as the concentration of reactive PN increased (Figure 3A, lanes 4, 7 and 10 respectively). The degraded PN may contain PN derivative, which is nitrite (1-4, 6,14-18). These compounds may react/interact differently toward polypeptides, thereby producing different electrophoretic patterns. Moreover, the progressive decrease in intensity was observed as the concentration of degraded PN increased (Figure 3B, lanes 3, 6 and 9). Interestingly, the preincubation with reactive PN generated distinctive bands at low molecular mass (Figure 3B, lanes 4 and 7).

Likewise, the band intensity increased as the concentration of degraded PN was increased (Figure 4, lane 3, 6 and 9). Whereas, a decrease in band intensity was observed upon the increased concentration of reactive PN (Figure 4, lanes 4, 7 and 10) in both the silver stain and Coomassie blue gels. The differences in the number and the location/site of N-nitrosated amino acid residues, and the nitrosated nitrogen atoms in the side chain of these acid residues (with the exception of lysine which contains only one nitrogen atom in ε-amino group), all may account for the distinctive electrophoretic patterns exhibited in the preincubated samples.

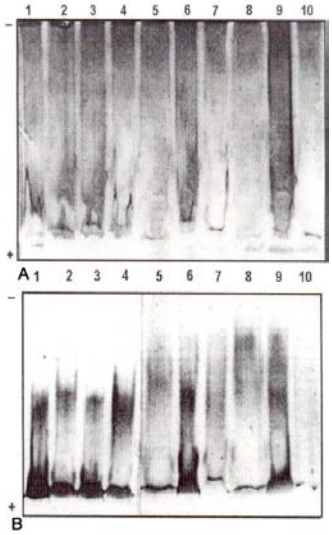

Figure 4. Modification of poly-histidine analyzed by SDS-PAGE with: **A.** Silver staining and **B.** Coomassie Blue G-250 staining. Lane 1, control, lanes 2, 5 and 8, controls with addition of degraded PN, 70, 140 and 210μM respectively (after preincubation) prior to SDS-PAGE; lanes 3, 6 and 9, preincubated with degraded PN 70, 140 and 210μM respectively; and lanes 4, 7 and 10, preincubated with reactive PN, 70, 140 and 210μM respectively.

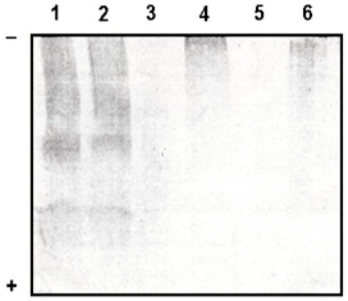

Figure 5. Probable N-nitrosation of histidine in PN-reacted histones detected by Western blot using anti-nitrosohistidine as a primary antibody. Lanes 1, 3, and 5 were controls for H1, H2A and H2B, respectively; lanes 2, 4, and 6 were PN-reacted H1, H2A and H2B, respectively.

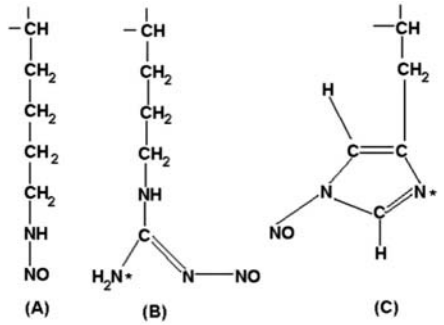

Figure 6. Probable sites of N-nitrosation of (A) ε-amino group of lysine, (B) guanido group of arginine and (C) imidazole group of histidine residues in polypeptides. Sites of alternative N-nitrosation with less probability and stability.

The probable nitrosation of histidine is partially supported by Western Blot analysis using anti-nitrosohistidine (Figure 5). An increase in immunoreactivity was observed when histone H2A and H2B were preincubated with PN (Figure 5, lanes 4 and 5 respectively). Since histidine is absent from histone H1 (19), the immunoreactivity of anti-nitrosohistidine observed in the sample of H1, with or without PN preincubation (Figure 5, lanes 1 and 2), may be due to nonspecific immunoreactivity. In addition, the discrepancy of decreased immunoreactivity was found in PN preincubated the H3 and H4 (data not shown). Furthermore, the N-nitrosation of PN-preincubated polylysine or polyhistidine was undetectable by Western blot analysis (data not shown).Therefore, a novel approach to produce highly specific antibodies is necessary to gain insight of N-nitrosations.

Covalent modifications on basic amino acids are less common, but nevertheless found in certain proteins. These modified amino acids include 3-methylhistidine, ε-N-methyllysine, ε-N-acetyllysine and N-methylarginine (20). Accordingly, the most probable sites of N-nitrosation on lysine, arginine and histidine side chains of polypeptides are similarly proposed in figure 6. However, the sites of nitrosation may also occur at a different nitrogen atom in the imidazole group of histidine residues or in the guanido group of arginine. It is possible that these alternative sites of nitrosation account for the difference in the sensitivity of Coomassie blue and silver staining observed in this study. Moreover, the varied intensity of nitrosation on peptides may result in the complexity of electrophoretic patterns mentioned above.

There are multiple modifications by PN and its derivatives on the amino acid residues of polypeptides. The stability of those modifications may be in the order of nitrotyrosine > aminotyrosine > S-nitrosocysteine > N-nitrosated amino acids (9). Despite the instability of nitroso groups, N-nitrosations may still play a role in the absorption of nitrosative stress and relaying NO signals (12). It may also be involved in 'transient' regulation of the N-methylation of histidine, arginine and lysine, and the acetylation of lysine.

5. ACKNOWLEGDEMENTS

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