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N-NITROSATION AND DENITROSATION ON LYSINE RESIDUES OF HISTONES

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1. ABSTRACT

Increased immunoactivity of ε-nitrosolysine was detected in Western blot in peroxynitrite-reacted histones H2A, H3 and H4.The N-nitrosation may be reversible as indicated by the decreased immunoactivity via either subsequent incubation with reduced glutathione or 48-hour dialysis and 48-hour storage at 4°C. Nonetheless, the biological significance of N-nitrosation of protein lysine is yet to be proven.

2. INTRODUCTION

The diverse biological role of nitric oxide (NO) has been extensively studied. In addition to many of the actions of NO via its ability to react with soluble guanylate cyclase and subsequently increased cyclic GMP level, a considerable portion of the biological function of NO are cyclic GMP-independent (1). The over activation and/or expression of nitric oxide synthase (2-6) can cause over production of NO which may react with superoxide free radicals to form peroxynitrite (ONO_2^-, PN), a potent oxidant (7-10), and subsequently be converted to nitrite/nitrate (3, 9, 11), these products may exert cGMP-independent effects. For example, it may cause undesirable effects such as PN-mediated oxidations and tyrosine-nitration (3, 10, 12, 13, 14) in oxidative stress and PN/nitrate/nitrite-mediated S-nitrosation of thiols (3, 12, 15) in nitrosative stress (11). It has been speculated that the formation of 3-nitrotyrosine, a stable product, may not only disturb the tyrosine-phosphorylation/dephosphorylation cascade (16, 17) but also alter the activities of a variety of enzymes (17-44).

It has also been postulated that NO might react with oxygen to form the nitrosating agent N_2O_3 at pH 7.0, thereby producing S-nitrosothiol (43). S-Nitrosation may modulate the function of many enzymes (44-48). Moreover, the occurrence of S-nitrosothiols in a variety of tissues may represent a novel class of signaling molecules (49) in addition to NO. Interestingly, nitrite can be easily protonated to form nitrous acid in extremely acidic conditions (pH < 3), and thus nitrosates thiols (3, 50). Surprisingly, regardless of less intensive reactions, S-nitrosation of thiols by nitrite may occur at neutral or physiological pH (15, 51). Both NO and PN are short-lived, but their metabolites, nitrite and nitrate, are more stable. It is noteworthy that the effects of PN/nitrite/nitrate are not necessarily always harmful. Instead, their actions with appropriate timing, sites and intensity could be beneficial, such as the direct and indirect involvement of PN and nitroso-thiols in the signalling process that could relay the effects of NO (15, 49, 51-53). Despite the remarkable progress in the research of tyrosine-nitration and thiol-nitrosation (S-nitrosation) of proteins, the other types of nitrosation of proteins still remain unanswered. Therefore, we explored the possible N-nitrosation and denitration on the ε-amino group of lysine residues in various histones.

3. MATERIALS AND METHODS

3.1. Materials

Reduced glutathione (GSH), sodium dodecyl sulfate (SDS) and EDTA were supplied by Sigma Chemical Company, St. Louis, MO, USA. Western blotting reagents, nitrocellulose membranes, anti-rabbit IgG alkaline phosphatase conjugate were obtained from Bio-Rad Laboratories, Hercules, CA, USA. Peroxynitrite was purchased from Upstate Biotechnology, Lake Placid, NY, USA. Histone HI and rabbit polyclonal anti-nitrosolysine was provided by Calbiochem-Novabiochem Corporation, San Diego, CA, USA. Histone H1, H2A, H3 and H4 was provided by Roche Molecular Biochemicals, Indianapolis, IN, USA.

3.2. Nitrosation of Histones

The incubation mixture was in a final volume of 36 µl of 50 mM potassium phosphate buffer (pH 7.0) containing approximately 36 µg of histone and 70 µM active peroxynitrite. The reactions were carried out at 25° C for 15 minutes. With or without subsequent incubation with 10mM GSH, the resulting samples were subject to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (12%) and Western blotting. In another experiment PN-

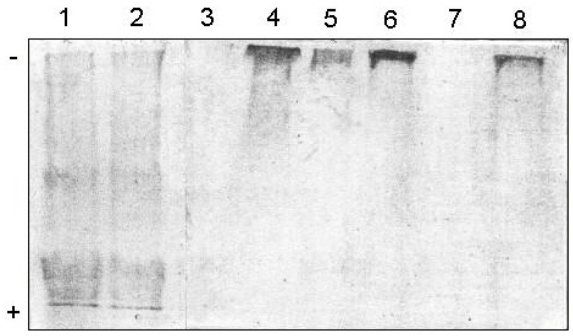

Figure 1. N-nitrosolysine of PN-reacted histones detected by Western blotting. Lanes 1, 3, 5 and 7 were histone H1, H2A, H3, and H4 respectively; lanes 2, 4, 6 and 8 were PN(70 μM)-reacted H1, H2A, H3 and H4, respectively.

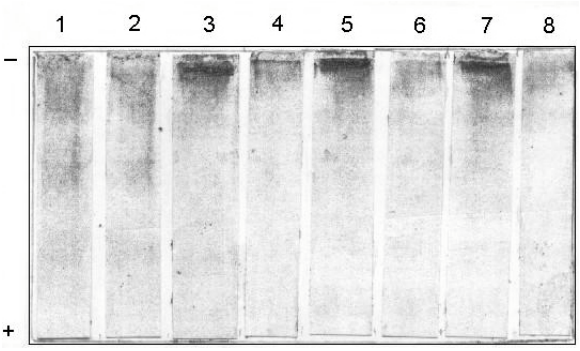

Figure 2. Decreased N-nitrosolysine immunoreactivity of PN- reacted histones due to subsequent incubation with GSH. Lanes 1, 3, 5 and 7 were PN(70 μM)-prereacted histones H1, H2A, H3 and H4, respectively; lanes 2, 4, 6 and 8 were PN-prereacted histone H1, H2A, H3 and H4 with subsequent GSH incubation.

reacted histones were dialyzed for 48 hours and stored at 4°C for an additional 48 hours before Western blot analysis.

3.3 Western blot analysis of nitrosated histones

With minor modifications, by the addition of 1mM EDTA in both tank and transfer buffers and the omission of the step of samples boiling prior to SDS-PAGE, the experimental procedures of Western blot basically followed those from past studies (12, 41) and was performed at 4°C. Bio-Rad's MiniPROTEIN 3 Cell was employed for both SDS-PAGE and protein blotting (to the nitrocellulose membrane). Experiments required pre-cooled tank buffer and prefrozen Towbin transfer buffer. Rabbit anti-nitrosolysine was used as the primary antibody (0.05 μg/ml), and anti-rabbit IgG-AP conjugate as the secondary antibody.

4. RESULTS AND DISCUSSION

Prior to SDS-PAGE, some modifications such as omitting the step of sample boiling in all experiments and omitting the reducing agent in some experiments were introduced in this study. However, these deviations from standard procedure of SDS-PAGE resulted in some difficulties to pinpoint accurately the molecular mass of immunoreactive bands in the Western blot. This modification was required to stabilize PN-reacted histones. Practically, these immunoreactive bands were distributed between 11 kDa and 20 kDa as the molecular mass of histones obtained commercially. Additionally, using cold buffer and performing SDS-PAGE and Western blot at 4°C had stabilized the nitroso group of PN-reacted histones as indicated by the clear immunoreactive bands (Figures 1-3).

Histones were selected as model proteins for this investigation due to their lysine-rich nature (54). PN-reacted histones H2A, H3 and H4 (Figure 1, lanes 4,6 and 8) had increased immunoactivity of nitrosolysine compared to unreacted histones (Figure 1, lanes 3,5 and 7). The N-nitrosation most likely occurred at ε-amino group of lysine residues of histone. Nevertheless, for unknown reasons increased immunoactivity of PN-reacted histone H1 (Figure 1, lanes 1 and 2) was not observed, despite the occurrence of a much greater number of lysine residues of H1 in comparison with those of H3, H4 and H2A (54). The intensity of nitrosolysine immunoactivity in the histone may not exclusively be decided by the number of lysine residues present in the histone but may also depend on the environment surrounding lysine residues such as the polarity, the steric hindrance and the overall conformation of the specific lysine-containing domain of the histone. Moreover, these lysine residues of histone H1 may have been endogenously nitrosated, and some of these N-nitroso groups may be relatively stable than those in histone H2A, H3 and H4.

It has been reported that S-nitrosothiols can be degraded by denitrosation in the presence of GSH (50, 12). Likewise, the degradation of N-nitroso group of PN-reacted histones after the incubation with 10 mM GSH may also occur as indicated by the decreased immunoreactivity of PN-reacted histones H2A (Figure 2, lane 4), H3 (Figure 2, lane 6) and H4 (Figure 2, lane 8).

After 48-hour dialysis and 48-hour storage at 4°C, decreased immunoactivity of PN-reacted histone was observed in all histone samples (Figure 3, lanes 2, 4, 6 and 8), suggesting that the N-nitroso groups at ε-lysine were degraded, presumably via denitrosation. These results suggest that the process of lysine N-nitrosation of histone/protein may be reversible under appropriate conditions, such as fast denitrosation in the presence of GSH and other reducing agents, whereas slow denitrosation in the absence of NO_2^-/NO_3^- via dialysis. In contrast, the nitrotyrosine residue of PN-reacted protein, e.g., nitrated bovine serum albumin, was found to have much greater stability after dialysis and 3-month storage. Additionally, nitrotyrosine was reduced into aminotyrosine after incubation with GSH (55, 17). Thus far, it lacks evidence to support the denitration of nitrotyrosine (into tyrosine) from the nitrated proteins.

The overall scheme of nitrosation by PN and the denitrosation either by dialysis and storage or reduction by GSH is illustrated in figure 4. The denitrosated product,

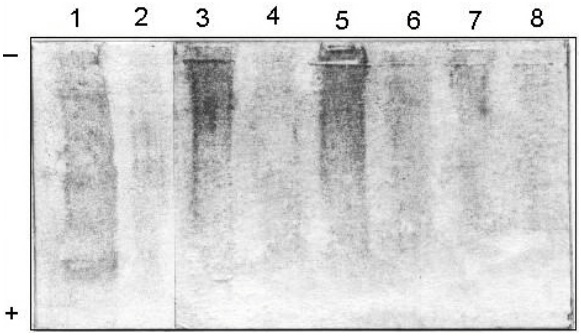

Figure 3. The degradation of N-nitroso group in the lysine residue of PN-reacted histones. Lanes 1, 3, 5 and 7 were PN-reacted histones H1, H2A, H3 and H4 respectively; lanes 2, 4, 6 and 8 were all of the above respective histones with subsequent 48-hour dialysis and 48-hour storage at 4°C.

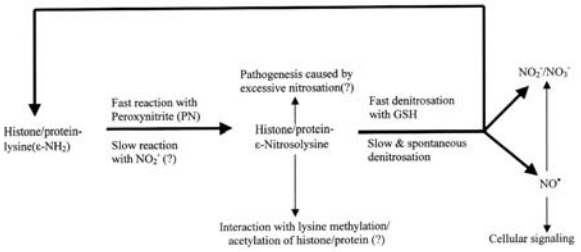

Figure 4. Schematic diagram of the possible effects of protein lysine N-nitrosation.

nitric oxide may be further utilized to relay cellular signal. In addition, NO_2^- might exert both an effect on N-nitrosation at slow pace and an inhibitory effect on the denitrosation. The N-nitrosation at the ε-NH_2 group of lysine residues of histones might be biologically significant. In addition to the lysine acetylation of histones H2B, H2A (56), H3 and H4 (57, 58) the lysine methylation of histone H3 and H4 (59) have been documented. The N-nitrosation, could occur at these lysine residues of histones, and trigger "cross talk" with methylation and/or acetylation, thereby providing a fine-tuning mechanism to regulate gene expression. Moreover, the ubiquitination at the ε-amino groups of lysine residues of a targeted protein (60) could be delayed/prevented by pre-nitrosation, should the protein still be needed and not badly damaged. Moreover, N-nitrosation may have impact on the activity of cyclic AMP-dependent protein kinase and the affinity of its substrate because the invariant lysine residue of subdomain II of the enzyme may assist in anchorage and orientation of ATP for phosphorylation (61).

5. ACKNOWLEDGMENTS

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