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Short communication

SHORT COMMUNICATION

Manganese superoxide dismutase (MnSOD) catalyzes NO-dependent tyrosine residue nitration

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Abstract: The peroxynitrite-induced nitration of manganese superoxide dismutase (MnSOD) tyrosine residue, which causes enzyme inactivation, is well established. This led to suggestions that MnSOD nitration and inactivation *in vivo*, detected in various diseases associated with oxidative stress and overproduction of nitric monoxide (NO), conditions which favor peroxynitrite formation, is also caused by peroxynitrite. However, our previous *in vitro* study demonstrated that exposure of MnSOD to NO led to NO conversion into nitrosonium (NO^+) and nitroxyl (NO^-) species, which caused enzyme modifications and inactivation. Here it is reported that MnSOD is tyrosine nitrated upon exposure to NO, as well as that MnSOD nitration contributes to inactivation of the enzyme. Collectively, these observations provide a compelling argument supporting the generation of nitrating species in MnSOD exposed to NO and shed a new light on MnSOD tyrosine nitration and inactivation *in vivo*. This may represent a novel mechanism by which MnSOD protects cell from deleterious effects associated with overproduction of NO. However, extensive MnSOD modification and inactivation associated with prolonged exposure to NO will amplify the toxic effects caused by increased cell superoxide and NO levels.

Keywords: MnSOD, nitric oxide, peroxynitrite, 3-nitrotyrosine.

INTRODUCTION

The nitrogen monoxide molecule (NO), a known environmental pollutant, is an ubiquitous cellular messenger, which regulates numerous biological processes, but its overproduction appears to contribute essentially to the pathology of disease. The pathophysiological effects are related to the generation of "reactive nitrogen species" (RNOS), such as oxides of nitrogen (NO_x) and peroxynitrite ($ONOO^-$), which are formed by the reaction of NO with oxygen and superoxide, respec-

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tively.[1] Of these, peroxynitrite, a strong oxidizing and nitrating agent, is now thought of as a potent mediator of NO induced cytotoxicity.[2,3]

Tyrosine nitration is a covalent posttranslational protein modification derived from the reaction of proteins with nitrating agents, which has been detected in a variety of disease states.[1,3] Protein nitration appears to be a selective process since not all tyrosine residues in proteins or all proteins are nitrated *in vivo*.[4,5] In addition to serving as a marker of reactive nitrogen species, nitration of tyrosine can severely compromise the function of proteins if the affected tyrosine residues are in the active site of the protein.[1,3]

The descovery that proteins at sites of cellular damage in diseases associated with oxidative stress bear heavily nitrated tyrosyl groups, together with the *in vitro* experiments which demonstrated that authentic peroxynitrite is an efficient nitrating agent, led to the suggestion that peroxynitrite could be a major tyrosine nitrating agent *in vivo*.[3,6,7] However, direct evidence for peroxynitrite-mediated nitration *in vivo* is still lacking.[8,9] Moreover, a number of recent *in vitro* studies demonstrated that the co-generation of NO and $O_2^{\bullet-}$, which is a better approximation to the *in vivo* situation than bolus addition of concentrated peroxynitrite solutions, did not cause significant nitration of tyrosine.[10–12] Several alternative pathways for nitrotyrosine formation under physiological conditions have been identified and data obtained up to now suggest that not only one reaction but rather a combination of pathways may be responsible for the nitration of tyrosine residues in proteins.[1,4]

Manganese superoxide dismutase (MnSOD) is the SOD isoform found in the mitochondrial matrix of eukaryotes and in a variety of prokaryotes. The enzymes from various sources have a high degree of structural similarity and contain identical metal chelating amino acid groups at the active site. MnSOD catalyzes the decomposition of superoxide and, therefore, plays an active role in both detoxification of the cell from this reactive oxygen species, and prevention of its reaction with NO to form peroxynitrite.[13,14] The essential nature of MnSOD was demonstrated by the discovery that deletion mutation of the MnSOD gene is mice resulted in death within 5–21 days of birth.[15,16] In different pathophysiologies associated with oxidative stress and overproduction of nitric oxide, MnSOD was reported to be tyrosine nitrated and inactivated.[17] Based on *in vitro* studies demonstrating that peroxynitrite inactivates MnSOD, causing nitration of critical enzyme tyrosine residue(s), it was suggested that peroxynitrite is the probable nitrating species causing MnSOD nitration *in vivo*.[18–21]

However, our previous *in vitro* study demonstrated that exposure of MnSOD (*E. coli*) to NO led to its fast and extensive inactivation, which was accompanied by structural alterations, such as amino acid group depletion and the cleavage of the polypeptide chains of the enzyme. The generation of nitrosonium (NO^+) and nitroxyl (HNO/NO^-) species in NO treated MnSOD, which produced enzyme modifications and inactivation, was demonstrated.[22] It is shown here that inactivation of NO treated MnSOD is associated with the tyrosine nitration of the enzyme. Colectively, these observations provide a compelling argument supporting the direct involvement of NO in the generation of nitrating species in MnSOD exposed to NO.

EXPERIMENTAL

All chemicals were reagent grade and used without additional purification. MnSOD (*E. coli*) was isolated according to Keele *et al.*[23] The protein concentration in the dissolved enzyme samples was determined according to Bradford *et al.*[24] The enzyme activity was assayed by the adrenalin method.[25] The enzyme preparations had typically a specific activity of 2300 units/mg. Manganese was removed from the active site essentially as described in Quijano *et al.*[21] Deionized Milli-Q water was used for washing glassware and preparing buffer solutions.

An argon purged solution (30 min) of MnSOD (15 μM enzyme monomer) in 50 mM potassium phosphate buffer pH 7.4 containing 100 mM EDTA (KPi), was exposed to a saturated (1.7 mM) solution of NO (15 min – 24 h, 23 °C) as described previously.[22] The remaining NO was subsequently removed by bubbling the medium with argon for 30 min.

Peroxynitrite was prepared and quantified as described by Beckman *et al.*[26] Working solutions of peroxynitrite were prepared by diluting stocks with 1M NaOH prior to use. Peroxynitrite was added (23 °C) at the final indicated concentations to MnSOD in KPi buffer, while vortexing.

The nitration of MnSOD was assayed spectrophotometrically. After the reaction had taken place, the pH of the solution was adjusted to 10–11 with 4 M NaOH, and the absorbance was recorded at 428 nm using ε (428 nm) = 4200 cm^{-1} dm^3 mol^{-1}.[27] Nitration of MnSOD was also studied by high performance liquid chromatography (HPLC). MnSOD samples were dialyzed extensively against distilled water until the dialyzate did not show any qualitative reaction (pink coloring) with Greiss reagent.[28] The resulting proteins were hydrolyzed under standard conditions (5.7 M HCl at 110 °C for 24 h) in a closed vial. The solution was dried using a rotary evaporator under vacuum. The residue was redissolved in 50 mM sodium phosphate buffer (pH 3.0) and analyzed by HPLC as described below. HPLC analysis was carried out on a Waters 1529 instrument with a Waters 2487 dual UV/VIS detector. 3-Nitrotyrosine (3-NTYR) was separated using a 5-μm Hypersil reverse phase C-18 column under isocratic conditions with a mobile phase consisting of 50 mM NaH_2PO_4 (pH 3.0) and HPLC-grade methanol (90/10; v/v). The absorbance was monitored at 365 nm.[19] The 3-NTYR peak was identified using an authentic external standard. The peak identity was determined by adding authentic 3-NTYR to the sample to establish the match in the HPLC retention time. The 3-NTYR concentration was calculated from the peak area using a calibration curve obtained with the standard. The data are expressed as nitrotyrosine/MnSOD subunit, obtained by dividing the total nitrotyrosine content by the molar concentration of MnSOD monomer.

RESULTS AND DISCUSSION

The HPLC analysis of hydrolyzed MnSOD which had been subjected to either peroxynitrite or NO treatment revealed a peak with the same retention time as the authentic 3-NTYR (Fig. 1). Mn-deficient superoxide dismutase did not form a nitrated adduct upon exposure to NO and was devoid of superoxide-scavenging activity.

Figure 2A shows that the 3-nitrotyrosine content of NO treated MnSOD increases with time and a maximum yield of one tyrosine residue per enzyme subunit could be predicted from these data. Figure 2B shows that tyrosine nitration parallels the loss of enzyme activity. Collectively, these data suggest that exposure to a high concentration of NO for a prolonged period of time is required for extensive modification and inactivation of MnSOD.

The peroxynitrite titration data (Fig. 3A) demonstrated that approx. three tyrosine residues in MnSOD were nitrated, as well as that one of these tyrosine residues is more susceptible to peroxynitrite induced nitration. All this is in good agreement with findings reported previously by other authors who used the same method and

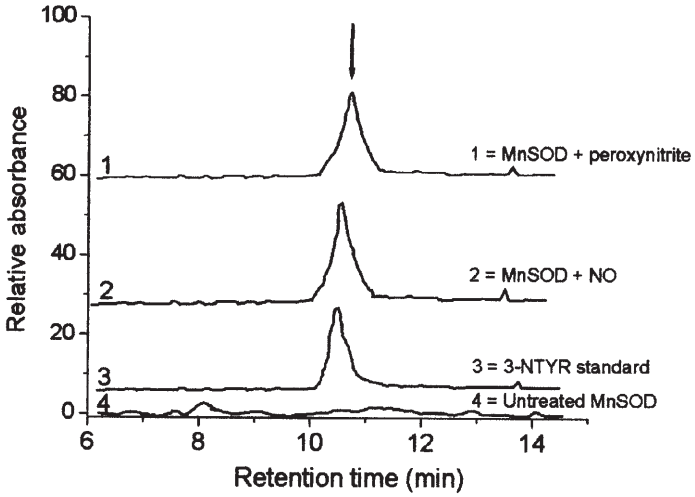

Fig. 1. HPLC detection of 3-nitrotyrosine formed in peroxynitrite (1) and NO-treated (2) MnSOD. Authentic 3-nitrotyrosine standard (3) and untreated MnSOD (4). An argon-purged solution of the enzyme (15 μM of enzyme monomer) in KPi buffer pH 7.4 was exposed to saturated (*ca*. 1.7 mM) NO solution at 23 °C for 15 min followed by bubbling the medium with argon for 30 min. Peroxynitrite (cumulative dose of 1.5 mM) was added sequentially to a stirred enzyme solution (15 μM enzyme monomer) in 50 mM KPi buffer, pH 7.4 at 23 °C. The MnSOD samples were dialyzed against distilled water until the reaction for nitrite was negative and then hydrolyzed in 5.7 M HCl at 110 °C for 24 h. Nitrotyrosine was analyzed by HPLC on reverse phase C-18 column under isocratic conditions with 50 mM sodium phosphate buffer (pH 3.0)/methanol (90:10; v/v) as the mobile phase and UV detection at 365 nm. The experiments were performed as described in the Experimental section.

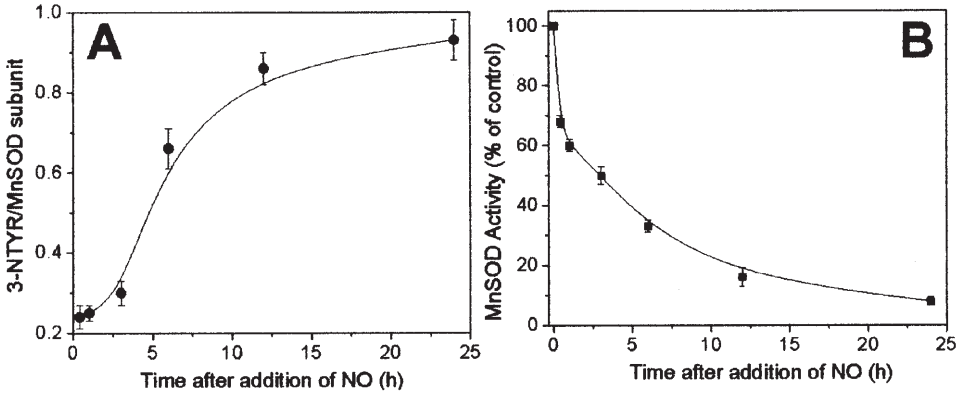

Fig. 2. Time-dependence of 3-nitrotyrosine formation (A) and enzyme inactivation (B) in NO-treated MnSOD. An argon purged solution of the enzyme (15 μM of enzyme monomer) in KPi buffer pH 7.4 was exposed to saturated (*ca.* 1.7 mM) NO solution at 23 °C for the indicated periods of time, followed by bubbling the medium with argon for 30 min. 3-NTYR was analyzed using the described HPLC method. Data are mean values ± S.D. of three experiments performed in duplicate. The experiments were performed as described in the Experimental section.

approach.[19] Consistent with previous findings,[19,20] peroxynitrite-induced enzyme inactivation parallels the nitration of one, and most likely the more susceptible, tyrosine residue (Fig. 3B).

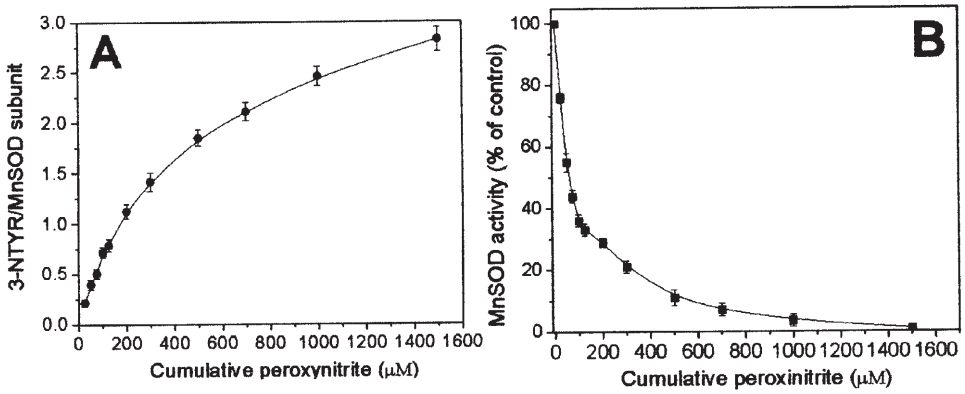

Fig. 3. Peroxynitrite concentration dependence of 3-nitrotyrosine formation (A) and MnSOD inactivation (B). Peroxynitrite was added sequentially to a stirred enzyme solution (15 μM enzyme monomer) in 50 mM KPi buffer, pH 7.4 at 23 °C. Each point represents the bolus addition of peroxynitrite; complete reaction of peroxynitrite occurred (*ca* 30 s) prior to the next addtion. The 3-nitrotyrosine content of MnSOD treated with peroxynitrite was calculated from the absorbance at 428 nm using an extinction coefficient ε (428 nm) = 4200 cm^{-1} dm^3 mol^{-1}. The experiments were performed as described in the Experimental section.

Taken together, these data suggest that the reactive species generated upon direct binding of NO to MnSOD are less invasive than peroxynitrite in producing enzyme modifications and inactivation.

Evidence was provided in a previous study that NO-treatment of MnSOD (*E. coli*) gives rise to reactive nitrosyl complexes which is associated with NO conversion into NO^+ and HNO/NO^- species, presumably according to the following reaction scheme:[22]

$$E_{ox} + NO \rightarrow E_{red} + NO^+$$

$$E_{red} + NO \rightarrow E_{ox} + NO^-$$

It was suggested that the NO, NO^+ and NO^- species are reminiscent of the redox forms of dioxygen, $O_2^{\bullet-}$, O_2 and O_2^{2-}, respectively.[29] Therefore, the term NO-dismutation for NO conversion into NO^+ and NO^- species was suggested.[30] A portion of the generated NO^+ and NO^- species are consumed in *in situ* reactions with amino acid residues from the enzyme molecules, which explains the modification(s) of the enzyme amino acid groups and cleavage of the polypeptide chains of the enzyme at the histidine residues of the active site of the enzyme observed in a previous study. The other portion takes part in reactions with molecules present in

the reaction medium, such as the reaction of NO^+ with water which yields nitrite, and the reaction of NO^+ and NO^- species with low molar mass thiols added to the reaction mixture yielding S-nitrosothiols and hydroxylamine, respectively.[22] The present study extends these findings by showing that reactive NO species capable of nitrating the tyrosine residues(s) of the enzyme are generated at the active site of NO treated enzyme.

It is assumed that NO treatment causes nitration of the Tyr34 residue of the enzyme molecule, which is located only a few angstroms from the manganese at the active site.[14,31] This tyrosine residue was shown previously to be the primary target for tyrosine nitration with peroxynitrate in MnSOD, which causes enzyme inactivation.[19,20] The Tyr34 residue is located at the vertex of the substrate funnel,[14,31] indicating that this residue may come in contact with nitrating species generated at the active site of the NO-treated enzyme.

The mechanism of the nitration of the tyrosine residue in NO-treated MnSOD is not yet clear. A pathway intiated by NO^+ derived C-nitrosation of the tyrosine residue followed by oxidation (with an as yet unidentified oxidizing agent) of the C-nitroso compound to the nitro derivative[32] seems plausible. However, a number of nitrite based reaction pathways of tyrosine nitration have recently been proposed.[1] One could speculate that NO^+ generated at the active site of the enzyme may be capable of reacting with accumulated nitrite (the product of the reaction of NO^+ with water), to form nitrogen dioxide ($NO_2^{\bullet}$), a documented tyrosine nitrating species.[1] Indeed , the nitration of MnSOD (Fig. 2A) parallels the formation of nitrite: it is lower at the beginning of the exposure of the enzyme to NO but increases as the amounts of nitrite in reaction mixture increase with time.[22] The mechanism of the NO-dependent MnSOD nitration of the tyrosine residue of MnSOD is currently being investigated in our laboratory.

The present study suggests that NO-induced MnSOD tyrosine nitration and inactivation may be relevant for *in vivo* conditions associated with an overproduction of NO during a prolonged period of time. Transition metal centers are the major targets for NO binding in biological systems,[33] suggesting that binding of NO to MnSOD is more specific than that of peroxynitrite, which has been shown, in addition to being rapidly decomposed at pH 7.4, to react with virtually all classes of biomolecules, including low molar mass antioxidants which are present in abundance in the cell.[2,3] Taken together, these findings shed new light on MnSOD tyrosine nitration and inactivation *in vivo* and indicate a novel mechanism by which MnSOD may protect cell from deleterious effects associated with an overproduction of NO. However, extensive MnSOD modifications and inactivation associated with prolonged exposure to NO will amplify toxic effects caused by increased cell superoxide and NO levels.

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ИЗВОД

МАНГАН-СУПЕРОКСИД-ДИСМУТАЗА (MnSOD) КАТАЛИЗУЈЕ NO-ЗАВИСНО НИТРОВАЊЕ ОСТАТКА ТИРОЗИНА

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Добро је познато да пероксинитрит изазива нитровање остатака тирозина у манган-супероксид-дисмутази (MnSOD) што доводи до инактивације ензима. Показано је да нитровање и инактивација MnSOD-а настаје у разним болестима, за које је карактеристичан оксидативни стрес и повећана продукција азот-моноксида (NO). Пошто се при овим условима очекује настајање пероксинитрита предложено је да пероксинитрит изазива нитровање и инактивацију MnSOD *in vivo*. У нашем претходном раду показали смо да MnSOD катализује трансформацију NO у нитрозонијум (NO^+) и нитроксил (NO^-) реактивне врсте, те идентификовали неке од модификација молекула ензима које при томе настају изазивајући његову инактивацију. У овом раду је показано да при излагању MnSOD азот-моноксиду долази и до нитровања остатка тирозина у молекулу ензима, што доприноси његовој инактивацији. Ови резултати указују да при интеракцији MnSOD са NO долази до настајања нитрујућих врста, што баца ново светло на процес нитровања остатака тирозина и инактивацију MnSOD *in vivo*. Ово може да представља нови механизам којим MnSOD штити ћелију од штетних ефеката изазваних хиперпродукцијом азот-моноксида. Међутим, екстензивне модификације и инактивација MnSOD до којих долази при продуженом излагању ензима NO, увећаће токсичне ефекте изазване повећаним концентрацијама супероксида и NO у ћелији.

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