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Consequences of MnSOD interactions with nitric oxide: Nitric oxide dismutation and the generation of peroxynitrite and hydrogen peroxide†

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Abstract

The present study demonstrates that manganese superoxide dismutase (MnSOD) (*Escherichia coli*), binds nitric oxide (˙NO) and stimulates its decay under both anaerobic and aerobic conditions. The results indicate that previously observed MnSOD-catalyzed ˙NO disproportionation (dismutation) into nitrosonium (NO^+) and nitroxyl (NO^-) species under anaerobic conditions is also operative in the presence of molecular oxygen. Upon sustained aerobic exposure to ˙NO, MnSOD-derived NO^- species initiate the formation of peroxynitrite ($ONOO^-$) leading to enzyme tyrosine nitration, oxidation and (partial) inactivation. The results suggest that both $ONOO^-$ decomposition and $ONOO^-$-dependent tyrosine residue nitration and oxidation are enhanced by metal centre-mediated catalysis. We show that the generation of $ONOO^-$ is accompanied by the formation of substantial amounts of H_2O_2. MnSOD is a critical mitochondrial antioxidant enzyme, which has been found to undergo tyrosine nitration and inactivation in various pathologies associated with the overproduction of ˙NO. The results of the present study can account for the molecular specificity of MnSOD nitration *in vivo*. The interaction of ˙NO with MnSOD may represent a novel mechanism by which MnSOD protects the cell from deleterious effects associated with overproduction of ˙NO.

Keywords: *Nitric oxide, MnSOD, nitroxyl, peroxynitrite, tyrosine nitration, hydrogen peroxide*

Introduction

Manganese superoxide dismutase (MnSOD) is the SOD isoform found in the mitochondrial matrix of eukaryotes and in a variety of prokaryotes. SOD enzymes from various sources have a high degree of structural similarity and contain identical metal-chelating amino acid groups in the active site [1,2]. The biological role of SOD is the detoxification of the superoxide radical ($O_2^{\cdot-}$) by converting it into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) [1,2].

MnSOD has been found to be inactivated and tyrosine nitrated in different pathologies associated with the overproduction of nitric oxide (˙NO) [3–12]. As MnSOD is a critical mitochondrial antioxidant enzyme, its nitration is thought to represent a severe hazard and has been suggested to promote oxidative damage, which may ultimately signal to cell death [3]. *In vitro* studies have demonstrated that synthetic peroxynitrite ($ONOO^-$) causes MnSOD tyrosine nitration and inactivation [13–15]. It has also been suggested that peroxynitrite $ONOO^-$, which can be

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†The authors dedicate this paper to Prof. A. M. Michelson on the occasion of his 80th birthday in 2006.

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formed in an extremely fast reaction between ˙NO and $O_2^{\cdot-}$ (second order rate constant of approximately $10^{10}\,M^{-1}\,s^{-1}$ [16]), causes MnSOD tyrosine nitration and inactivation *in vivo* [13,14].

Previous studies from our laboratory [17] have demonstrated that in anaerobic solutions MnSOD (*Escherichia coli*) catalyses ˙NO conversion (disproportionation) into nitrosonium (NO^+) and nitroxyl anion (NO^-) species (reactions 1–2), which lead to structural enzyme modifications and inactivation [17]. The term ˙NO-dismutation [18] was suggested to distinguish this from other modes of ˙NO disproportion promoted by transition metal complexes [19].

We hypothesised that if MnSOD-catalysed ˙NO dismutation was operative under aerobic conditions (O_2 may react with ˙NO [20] thereby preventing its interaction with the enzyme) NO^- derived from MnSOD-catalysed ˙NO disproportionation (reaction 2) could trigger $ONOO^-$ formation via its rapid reaction with O_2 [21] (rate constant of $5.7 \times 10^7\,M^{-1}\,s^{-1}$ [22]) (reaction 3). The $ONOO^-$ formed by such a mechanism would be generated at/or near the enzyme's active site. Therefore, both $ONOO^-$ decomposition and $ONOO^-$-dependent tyrosine residue nitration and oxidation would be enhanced by metal centre-mediated catalysis [15].

$$\text{Mn(III)SOD} + {}^{\cdot}\text{NO} \rightarrow \text{Mn(II)}\cdots\text{NO}^+ \qquad (1)$$

$$\text{Mn(II)SOD} + {}^{\cdot}\text{NO} \rightarrow \text{Mn(III)}\cdots\text{NO}^- \qquad (2)$$

$$\text{NO}^- + \text{O}_2 \rightarrow \text{ONOO}^- \qquad (3)$$

Here, we demonstrate that MnSOD (*E. coli*) binds and stimulates ˙NO decay under both anaerobic and aerobic conditions. We show that upon sustained aerobic exposure to ˙NO, MnSOD-derived NO^- initiates the formation of $ONOO^-$ which leads to $ONOO^-$ decomposition and $ONOO^-$-dependent enzyme tyrosine residue nitration, oxidation and (partial) enzyme inactivation. Substantial amounts of H_2O_2 generated upon aerobic MnSOD exposure to ˙NO support the intermediacy of $O_2^{\cdot-}$ during the reaction of MnSOD-derived NO^- with O_2. The data suggest a novel protective role of MnSOD and can account for the molecular specificity of MnSOD nitration *in vivo*.

Materials and methods

Reagents

MnSOD (from *E. coli*) was isolated according to Ref. [23]. Mn removal from the enzyme's active site and the preparation of the apoenzyme was performed as described in Ref. [15]. The enzyme activity was assayed by the adrenalin method [24]. $ONOO^-$ was synthesised, quantitated and handled as described in Ref. [25]. A dityrosine (dityr) standard was prepared using horseradish peroxidase-catalysed oxidation of tyrosine by H_2O_2 [26] and quantified using the extinction coefficient $E_{315} = 8380\,M^{-1}\,cm^{-1}$ [26]. Tyrosine and 3-nitro-L-tyrosine were purchased from Sigma. Human haemoglobin was isolated from red blood cells from healthy volunteers as previously described [27]. Methaemoglobin (MetHb) was prepared by oxidation of haemoglobin with excess sodium hexacyanoferrate(III) [27] and further purified by extensive dialysis using ultra-pure Milli-Q water. All other chemicals were at least analytical grade and were used without additional purification. Potassium phosphate (KPi) buffer (50 mM, pH 7.4) was prepared from K_2HPO_4/KH_2PO_4 using ultra-pure Milli-Q water and treated with the heavy metal scavenger resin Chelex-100 (0.3–0.5 g per 10 ml) by shaking gently for 18 h in the dark. After low-speed centrifugation for 15 min, the solution was carefully decanted from the resin and the pH of solution was checked and re-adjusted at 23°C. Gaseous ˙NO was obtained from the reaction of $FeSO_4$ with $NaNO_2$ in 0.1 M HCl and subsequently purified by passing through 0.5 M NaOH and then through a solid $CaCl_2$ column [28]. ˙NO solutions (1.2–1.8 mM) were prepared by collecting gaseous ˙NO in argon (Ar)-purged glass vials fitted with septum containing KPi buffer. Solutions of low ˙NO concentrations were prepared by bubbling Ar through the millimolar ˙NO solutions until concentrations of between 10 and 150 μM of ˙NO were achieved. For ˙NO measurements, 50 μl aliquots of ˙NO solutions were sampled with a gas-tight Hamilton syringe and added directly to the stirred reaction chamber containing 5 ml of KPi buffer containing a ˙NO-sensitive electrode (Harvard Apparatus, USA).

Kinetics of MnSOD-stimulated ˙NO decay

An anaerobic ˙NO solution (10–150 μM, 2 ml) in KPi buffer was incubated for 60 min at 23°C before a further 60 min incubation which followed the injection of an Ar-purged stock solution of MnSOD. ˙NO was determined by sampling 50 μl aliquots of ˙NO solutions using a gas-tight Hamilton syringe at specific time points and then added directly to a stirred reaction chamber containing 5 ml of KPi buffer containing a ˙NO-sensitive electrode. At the end of the experiment, ˙NO was removed by bubbling Ar through the solution and the remaining enzyme activity was estimated.

The effect of MnSOD on the decay of ˙NO in an air-saturated buffer was estimated as described in Ref. [29]. Briefly, a ˙NO solution (1.6 mM) in KPi buffer was sampled with a gas-tight Hamilton syringe and added directly to the stirred reaction chamber containing 5 ml of air-saturated KPi buffer in the

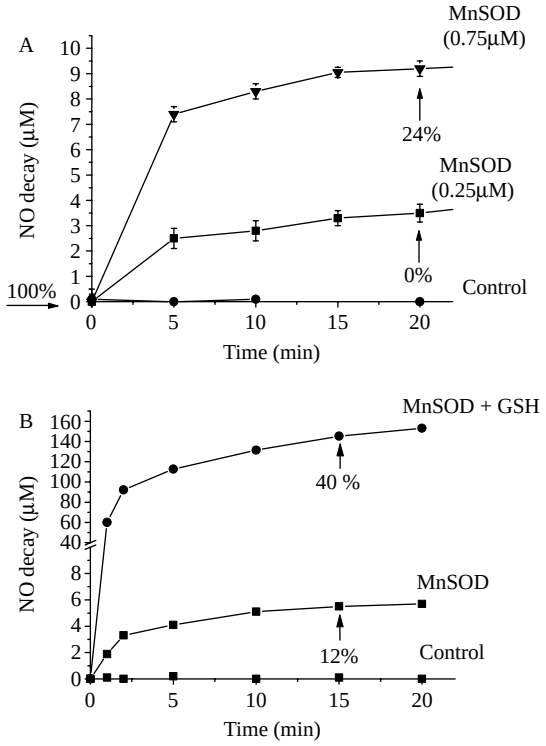

Figure 1. MnSOD causes rapid ·NO decay in anaerobic solution. (A) Anaerobic ·NO solution (10 μM) in KPi buffer was incubated for 60 min at 23°C and subsequently for a further 20 min following the injection of an Ar-purged stock solution of MnSOD to the final indicated enzyme monomer concentrations. (B) Anaerobic ·NO solution (150 μM) in KPi buffer was incubated with MnSOD (0.45 μM subunit) with and without GSH supplementation (1 mM). For ·NO measurments 50 μl aliquots of ·NO solutions were sampled with a gas-tight Hamilton syringe at the indicated time points and added directly to the stirred reaction chamber containing 5 ml of KPi buffer containing a ·NO-sensitive electrode. Arrows indicate the remaining enzyme activity as % of the control. Data are representative of at least three individual experiments.

absence and presence of MnSOD (5–25 μM) containing a ·NO electrode.

Stoichiometric determinations

To quantitate the reaction products formed upon aerobic MnSOD exposure to ·NO, aliquots of ·NO solution of known concentration (to yield a ·NO concentration of approximately 10 μM in the reaction mixture) were injected sequentially at 5 min intervals into a solution of MnSOD in air-saturated KPi pH 7.4 buffer at 23°C. Under these conditions, completed reactions of ·NO occurred (within 5 min) prior to the subsequent addition. Control experiments with the SOD apoenzyme were performed to estimate the potential effects of reactive ·NO species derived from the auto-oxidation of ·NO.

To assess the formation of NO^- and (under anaerobic conditions) NO^+, the MnSOD solution was supplemented with GSH (1 mM) prior to the addition of ·NO. Hydroxylamine and GSNO were estimated in the reaction mixture. Hydroxylamine was determined by measuring indooxine formation from 8-hydroxyquinoline [22]. Since the presence of thiols causes decreased colour yield, a standard curve was performed using solutions containing hydroxylamine and GSH to compensate for the decreased colour yield [22]. The formation of NO^-, upon aerobic MnSOD exposure to ·NO was assessed by reductive nitrosylation of metHb to HbNO which was monitored directly in a quartz cuvette in the assay buffer at 23°C [22]. GSNO was measured by the method of Saville [30].

The oxidation of non-fluorescent dihydrorhodamine-123 (DHR) to fluorescent rhodamine-123 (RH) was measured using a Fluorolog 3-22 spectrofluoimeter (Jobin Yvon, USA) with $\lambda_{excitation} = 503$ nm and $\lambda_{emission} = 526$ nm. The formation of RH was also quantified spectrophotometrically at 500 nm ($\varepsilon = 78{,}000\ M^{-1}\,cm^{-1}$) [31]. Known amounts of synthetic $ONOO^-$ were added for calibration and to determine the relationship between $ONOO^-$ and DHR conversion into RH.

H_2O_2 was quantified spectrophotometrically by measuring the coloured product formed by peroxidase-catalysed oxidation of 4-aminoantipyrine [32].

Protein-bound 3-nitrotyrosine (3-NT) and dityr were determined by HPLC analysis using hydrolysed protein samples. To avoid artifactual nitration reactions during acid hydrolysis, due to the presence of nitrite in the reaction mixture, protein samples were extensively dialysed against ultra-pure Milli-Q water until the dialysate did not show any qualititative reaction (pink coloring) with the Griess reagent [33]. Protein samples (1 mg) were hydrolysed in sealed (Ar) glass ampoules under standard conditions (containing 1.5 ml of 5.7 M HCl at 110°C for 24 h). After drying the hydrolyzates under vacuum, the mixtures were resuspended in the appropriate mobile phase. 3-NT and dityr were analyzed by HPLC, using an ODS Hypersil column (200 × 4.6 mm, 5-μM diameter particles). Tyrosine, dityr, and 3-NT were eluted isocratically at 1 ml/min using a mobile phase consisting of 92% water, 8% acetonitrile and 0.1% trifluoroacetic acid [26] and analysed by UV detection at 274 nm. Data are expressed as 3-NT/MnSOD and dityr/MnSOD subunit. To determine the yields of 3-NT and dityr during acid hydrolysis and HPLC separation, the hydrolysis of MnSOD (1 mg) plus 50 nmol of each standard (3-NT and dityr) was performed in parallel. HPLC analysis revealed a 100% recovery of 3-NT and a 90% recovery of dityr under our conditions. Protein solutions were also analysed for the presence of dityr using a spectrofluorimeter. Aliquots of ·NO-treated or untreated MnSOD were added to solutions of 0.1 M KPi buffer (pH adjusted to 9.0) and the fluorescence emission was scanned

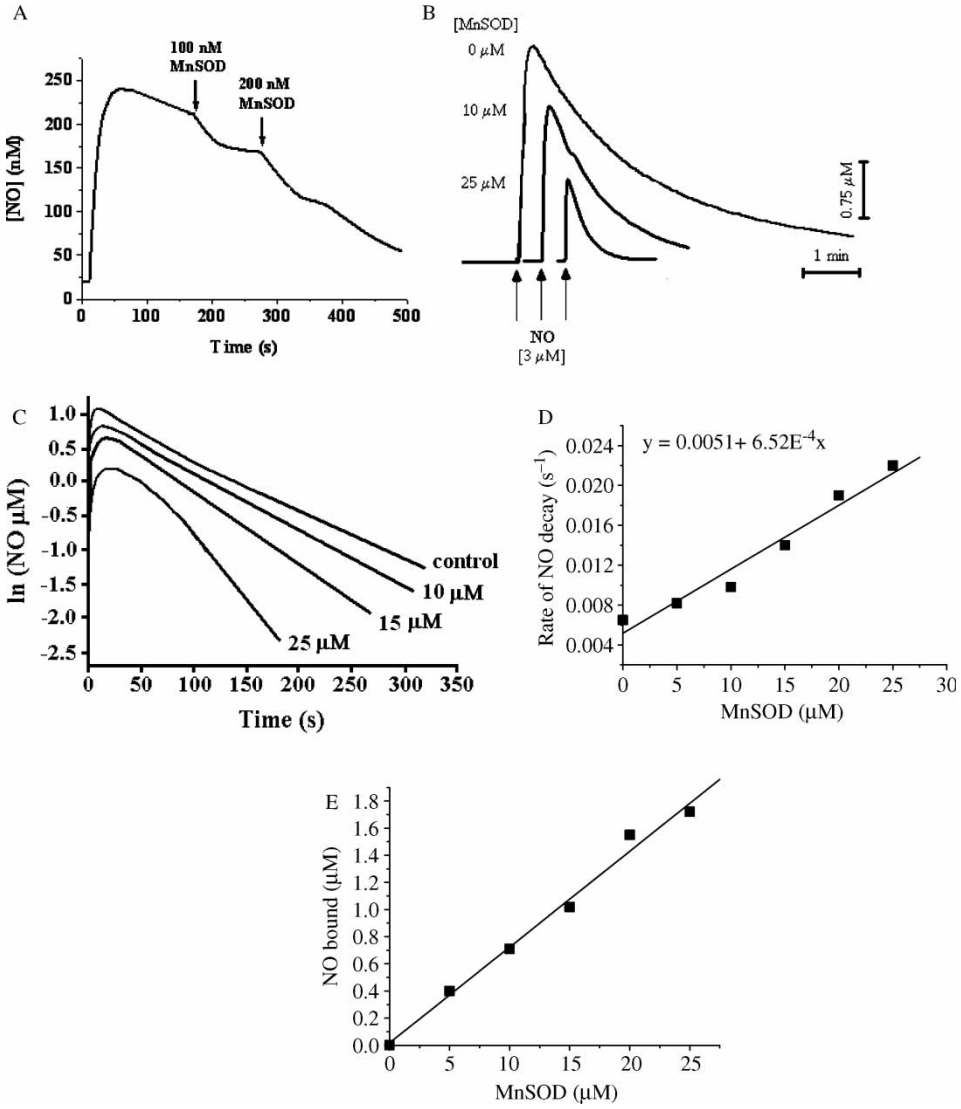

Figure 2. MnSOD catalyses ˙NO decay in air-saturated buffer. (A) A representative trace of ˙NO decay following the addition of MnSOD into the ˙NO solution (monitored with an ˙NO electrode). (B–D) The effect of adding 3 μM of ˙NO to aerobic solutions of increasing concentrations of MnSOD. Representative ˙NO traces (B), logarithmic plots (C) and the rate of ˙NO decay as a function of the initial MnSOD concentration (D). A plot of the binding of ˙NO as a function of MnSOD concentration: the intercept of the linear portion of the logarithmic plot with the time-zero point was used to calculate the amount of ˙NO rapidly removed from the solution and this is plotted as a function of the MnSOD (subunit) concentration (E). Data are representative of at least three individual experiments.

from 350 to 500 nm using a fixed excitation wavelenghth of 325 nm [13].

To be absolutely sure of the accuracy of nitrate estimation, the concentration of nitrite present in the reaction mixtures was first measured using the Griess reagent [33], followed by the addition of an equal amount of ammonium sulfamate dissolved in 50 mM HCl to remove the nitrite from the reaction mixtures. The nitrate concentration in the reaction mixtures was then estimated after its conversion to nitrite by means of the nitrate reducing wire (Innovative Instruments, USA) as described by the manufacturer.

˙NO-treated samples of MnSOD and the corresponding controls were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% running gel under reducing conditions [34].

Results

MnSOD stimulates ˙NO decay and ˙NO dismutation upon anaeorobic exposure to ˙NO

Figure 1A shows that addition of an Ar-purged solution of MnSOD into an anaerobic solution of ˙NO caused a rapid breakdown of ˙NO that was associated with enzyme inactivation. We can estimate that a minimum of 2 and 8 μM of ˙NO (of the total 10 μM added) was broken down by 0.25 and 0.75 μM of

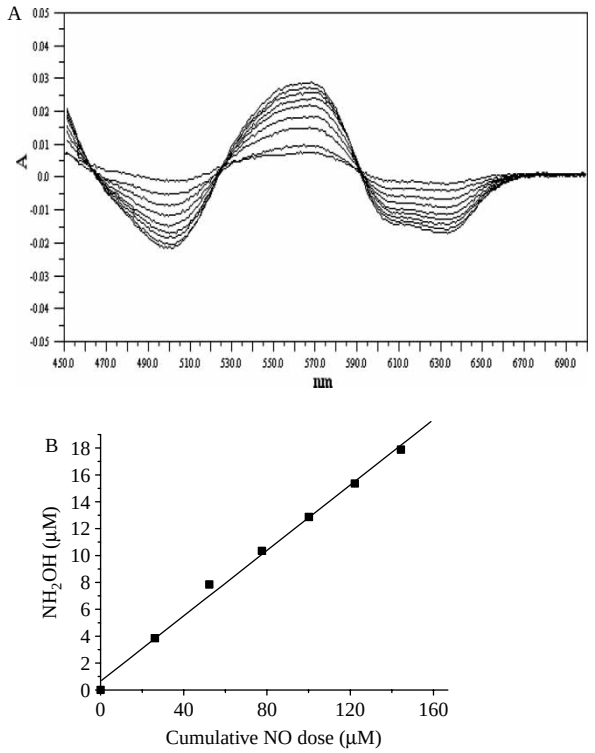

Figure 3. MnSOD-catalysed generation of NO^- upon aerobic exposure to ˙NO. (A) Reductive nitrosylation of MetHb (50 μM; 502 and 630 nm) to HbNO (543 and 582 nm). A solution of MnSOD (15 μM subunit) in air-saturated KPi buffer supplemented with MetHb was subjected to sequental bolus additions (each 10 μl) of saturated ˙NO solution (1.6 mM) to yield the ˙NO concentration in the reaction mixture of approximately 10 μM at 23°C. After each addition of ˙NO, the solution was incubated for 5 min. Under these conditions, completed reactions of ˙NO occurred (within 5 min) prior to the subsequent addition. The illustrated spectra were recorded after two additions of ˙NO solution. In control incubations (without MnSOD addition), ˙NO did not produce any effect on MetHb. (B) Estimation of hydroxylamine. A solution of MnSOD (15 μM subunit) supplemented with GSH (1 mM) was subjected to bolus additions of ˙NO as described in (A). After 2–3 additions of ˙NO solution (at the indicated cumulative ˙NO doses), aliquots of the reaction mixture were removed for hydroxylamine estimation. Data (A) are representative of at least three individual experiments. Data points (B) are the means from triplicate values representative of three separate experiments; SEM error bars were of similar size to the symbols and are thus omitted for clarity.

MnSOD, respectively suggesting that the reaction was in fact catalytic. These results are consistent with the previous finding that MnSOD (under anaerobic conditions) catalyses ˙NO disproportionation into NO^+ and NO^- species (reactions 1–2) causing structural enzyme modifications and catalytic inactivation [17]. Furthermore, the addition of GSH, which reacted with both NO^+ and HNO/NO^- species yielding GSNO (approximately 40 μM) and hydroxylamine (approximately 20 μM) respectively [22,30] significantly protected MnSOD from ˙NO-mediated inactivation thereby increasing the amount of ˙NO broken down by the enzyme (Figure 1B). In a control incubation with GSH (but without MnSOD), ˙NO decay was almost non-detectable (Figure 1B) which therefore excluded the reaction of GSH with ˙NO [35] as a source of ˙NO breakdown.

MnSOD stimulates ˙NO decay in the presence of oxygen

Since ˙NO decomposes slowly in an air-saturated buffer due to its reaction with O_2 [20], the capacity of MnSOD to increase ˙NO decay was examined. Results in Figure 2 show that MnSOD increased the rate of ˙NO breakdown in the presence of O_2. The effects of adding an aliquot of MnSOD solution into the aerobic solution of ˙NO and representative traces of ˙NO decay upon addition of ˙NO (3 μM) to the aerobic solution of MnSOD are shown in panels A and B, respectively. The effect of increasing concentrations of MnSOD on ˙NO (3 μM) decay (plotted logarithmically) is shown in panel C. Panel D shows that in the presence of increasing concentrations of MnSOD, the rate of ˙NO decay increased. Although the auto-oxidation of ˙NO by O_2 is a second-order process [20], under these conditions (low ˙NO concentrations and fixed high O_2 concentrations), it may be assumed that the rate approximates a first-order process [29]. Assuming that pseudo-first-order conditions are applicable at high MnSOD concentrations, the slope of this graph should yield the rate constant for the reaction of MnSOD with ˙NO. It was found to be approximately 650 $M^{-1} s^{-1}$. Extrapolating the linear segment of the logarithmic plot to zero time revealed a rapid decrease in the initial concentration of free ˙NO as the MnSOD concentration increased. This fraction was proportional to the MnSOD concentration and represented approximately 7% of the amount added (panel E).

MnSOD-stimulated ˙NO dismutation upon aerobic exposure to NO: Generation of NO^- species

If MnSOD-stimulated ˙NO decay in the presence of oxygen was due to MnSOD-catalysed ˙NO dismutation (reactions 1–2), it should therefore be possible to identify the generation of NO^- species in the reaction mixture. Indeed, both the reductive nitrosylation of MetHb to form HbNO [22] (Figure 3A) and thiol-dependent formation of hydroxylamine [22] (Figure 3B) strongly support our claim that MnSOD stimulated the formation of NO^- species upon sustained aerobic exposure to ˙NO.

Formation of $ONOO^-$ and H_2O_2 upon aerobic MnSOD exposure to ˙NO

The formation of MnSOD-derived NO^- species raised the possibility that $ONOO^-$ was formed upon the reaction of NO^- with O_2 (reaction 3). Indeed, Figure 4A shows that upon sustained aerobic MnSOD exposure to ˙NO DHR oxidation [31] occured in

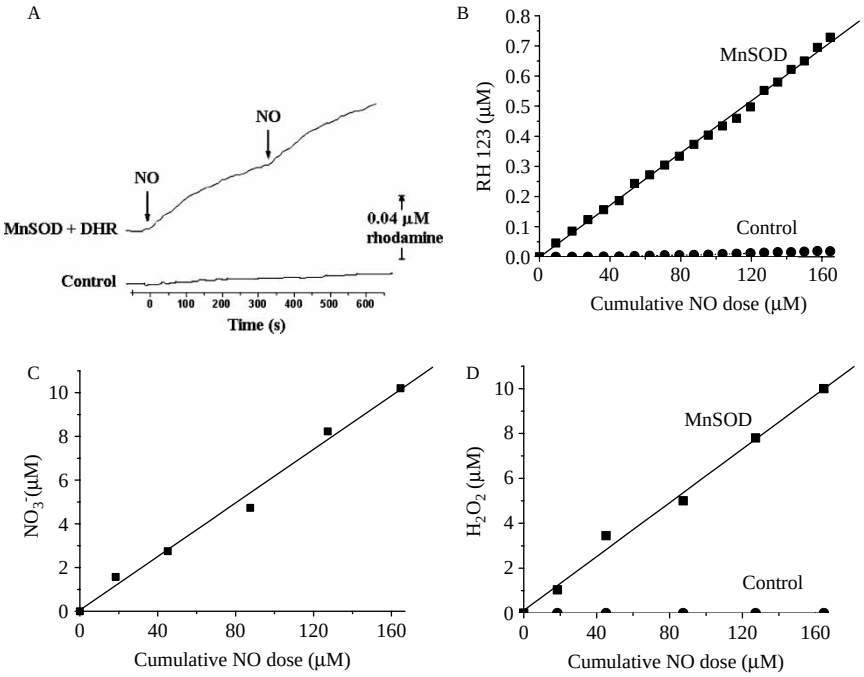

Figure 4. DHR oxidation and the formation of nitrate and H_2O_2 upon aerobic MnSOD exposure to ˙NO. (A) A representative fluorometric trace of DHR oxidation into RH. (B) The ˙NO concentration-dependant oxidation of DHR. A solution of MnSOD (15 μM subunit) and DHR (50 μM) in air-saturated KPi buffer at 23°C was subjected to sequential bolus additions of ˙NO solution as described in the legend to Figure 3. Control incubations were without MnSOD and were run in parallel. (C) The production of nitrate and (D) the production of H_2O_2 as a function of ˙NO added to the air-saturated buffer containing MnSOD (15 μM subunit). After 2–3 additions of ˙NO solution (at the indicated cumulative ˙NO doses) aliquots of the reaction mixture were removed for nitrate and H_2O_2 estimation. In control incubations (without MnSOD addition), nitrate was not detected. Nitrate and H_2O_2 were estimated as described in the Materials and Methods section.

parallel (at approximately 5 min) with the loss of ˙NO. DHR oxidation (50 μM) following sequental additions of ˙NO into aerobic MnSOD solution (as a function of increasing cumulative ˙NO dose, 0–165 μM) is shown in Figure 4B.

The addition of synthetic $ONOO^-$ oxidised DHR with an efficiency of approximately 60%, agreeing with a previously reported value [31]. In contrast, the addition of synthetic $ONOO^-$ to a MnSOD solution resulted in less oxidised DHR (approximtaely 5% efficiency), which may be explained by the MnSOD-catalysed decomposition of $ONOO^-$ demonstrated in a previous study [15].

A recent observation suggested that at neutral pH the protonated HNO form of NO^- predominates [36]. The oxidation of HNO forming a strong oxidant species (distinct from $ONOO^-$) capable of oxidising DHR has been reported [37,38]. Nitrate, which is the $ONOO^-$ decomposition product [39], was not detected among the end products of auto-oxidation of HNO [39] or small amount was detected upon completion of the reaction [40]. However, Figure 4C shows that substantial amounts of nitrate were found in the ˙NO-treated MnSOD reaction mixture. Together these results suggest that upon aerobic MnSOD exposure to ˙NO $ONOO^-$ was clearly generated in the reaction of MnSOD-derived NO^- species with O_2. However, the present data does not exclude the possibility that a portion of the MnSOD-derived NO^- species was protonated to form HNO which could be subsequently oxidised into a strong oxidant chemically distinct from that of $ONOO^-$ [37,38].

The formation of $ONOO^-$ in the reaction of NO^- with O_2 has been suggested to take place via the intermediate production of $O_2^{\cdot-}$, which is converted into H_2O_2 in the presence of SOD [31] (reactions 4–6). Accordingly, Figure 4D shows that substantial amounts of H_2O_2 (that approximate to the amounts of nitrate from Figure 4C) were generated upon aerobic MnSOD exposure to ˙NO.

$$NO^- + O_2 \rightarrow {}^{\cdot}NO + O_2^{\cdot-} \quad (4)$$

$$O_2^{\cdot-} + 2H^+ + SOD \rightarrow H_2O_2 + SOD \quad (5)$$

$$^{\cdot}NO + O_2^{\cdot-} \rightarrow ONOO^- \quad (6)$$

MnSOD tyrosine residue nitration, oxidation and enzyme inactivation upon aerobic enzyme exposure to ˙NO

The generation of $ONOO^-$ by the above-described mechanisms occuring at/or near the MnSOD active site raises the possibility that $ONOO^-$-dependent enzyme tyrosine residue nitration and oxidation could

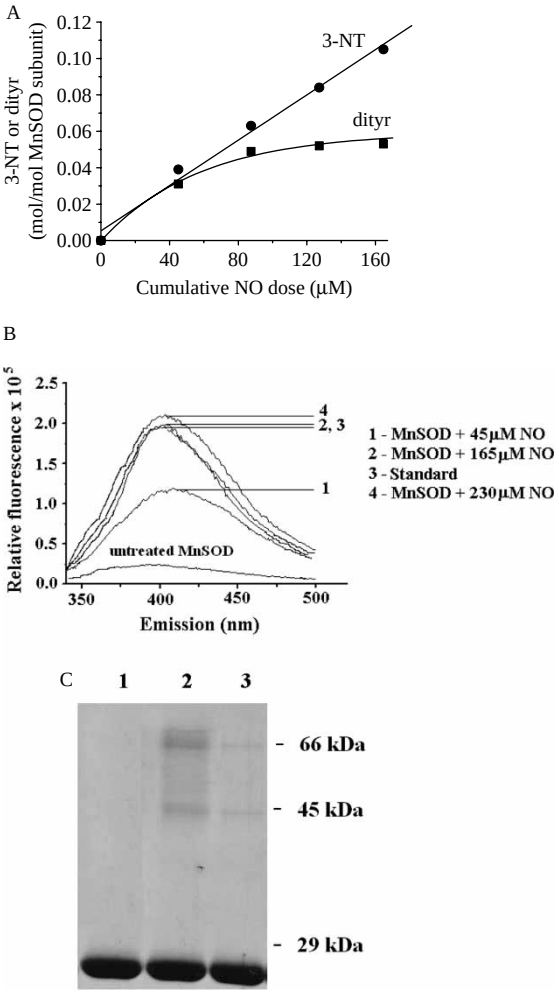

Figure 5. The 3-NT and dityr content of ˙NO-treated MnSOD as a function of ˙NO. MnSOD was exposed to ˙NO as described in the legend to Figure 3. (A) 3-NT and dityr (mol/mol MnSOD subunit) in hydrolysed MnSOD samples analysed by HPLC. (B) Fluorescence spectroscopy of intact proteins. Aliquots of ˙NO-treated or untreated MnSOD were added to solutions of 0.1 M KPi buffer (pH adjusted to 9.0) and the fluorescence emission was monitored from 350 to 500 nm at a fixed excitation wavelength of 325 nm. A synthetic dityr standard (0.75 μM) was used. (C) SDS-PAGE of ˙NO-treated MnSOD. Lane 1, control MnSOD; lanes 2 and 3, ˙NO treated MnSOD (cumulative ˙NO dose 165 and 45 μM, respectively).

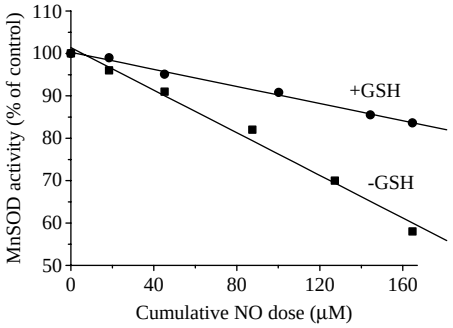

Figure 6. The effect of ˙NO treatment on MnSOD activity. Solutions of MnSOD (15 μM subunit) supplemented without or with GSH (1 mM) were exposed to ˙NO as described in the legend to Figure 3. Aliquots were withdrawn at the indicated time points for MnSOD activity measurements. Results from a single experiment (repeated three times) are shown.

be enhanced by metal centre-mediated catalysis [15]. HPLC analysis of hydrolysed MnSOD samples subjected to ˙NO treatment (under the conditions similar to those in Figure 3) revealed the presence of both 3-NT and dityr. The amount of 3-NT increased with the ˙NO dose, whereas the initial increase of dityr did not further increase despite continued addition of ˙NO (Figure 5A). Spectrofluorimetric analysis of ˙NO-treated samples to detect dityr (Figure 5B) confirmed the results from the HPLC analysis. SDS-PAGE indicated that ˙NO treatment resulted in the formation of non-reducible high molecular weight structures (Figure 5C). The simultaneous appearance of dityr and high molecular-mass complexes suggests that dityr promotes intermolecular covalent crosslinking between MnSOD subunits.

When Mn-deficient SOD apoenzyme or BSA was incubated with ˙NO (a cumulative dose of 165 μM) neither 3-NT nor dityr could be detected (data not shown), which excluded the auto-oxidation of ˙NO [41] as the source of tyrosine nitration and oxidation in ˙NO-treated MnSOD under our experimental conditions.

The majority of ˙NO added into a solution of MnSOD is converted to nitrite, a product of ˙NO auto-oxidation [20]. Given the ability of nitrite/H_2O_2 and metals to nitrate tyrosine [41], we incubated MnSOD (15μM of enzyme monomer) with nitrite and H_2O_2 (each 1 mM) for 2 h at 23°C. This treatment yielded approximately 0.01 mol of 3-NT per mole of the enzyme monomer. This suggested that under the experimental conditions of Figure 5 nitrite and H_2O_2 formed upon MnSOD exposure to ˙NO could not be the major source of enzyme tyrosine nitration.

Taken together our findings support the idea that $ONOO^-$ generated at or near the enzyme's active site upon aerobic MnSOD exposure to ˙NO causes tyrosine residue nitration and oxidation of the enzyme.

Treatment of MnSOD with specific cumulative ˙NO concentrations confirmed a dose-dependent inhibition of MnSODs enzymatic activity (Figure 6). However, in contrast to MnSOD treatment with synthetic $ONOO^-$, in which 0.25 tyrosine residue nitration per enzyme subunit was associated with partial enzyme inactivation [42], ˙NO-mediated MnSOD inactivation (approximately 40%) shown in Figure 6 was due to modification of 0.15 tyrosine residues per enzyme subunit (Figure 5). This means that when MnSOD is treated with ˙NO other pathways are operative in addition to $ONOO^-$-mediated enzyme inactivation. These include enzyme inactivation due to modifications of the enzyme molecule itself by NO^+ and NO^- species generated by MnSOD-stimulated ˙NO

dismutation observed in our previous study [17]. GSH, which reacts with both NO^+ and NO^- species [22,30], prevented both the reaction of NO^+ and NO^- species with MnSOD and NO^--mediated $ONOO^-$ formation protected MnSOD from inactivation (Figure 6) and inhibited tyrosine residue modification (data not shown).

Discussion

Our present study demonstrates that MnSOD (from *E. coli*) binds and catalyses ˙NO decay under both anaerobic and aerobic conditions. In addition, MnSOD-catalysed ˙NO dismutation (reactions 1 and 2) observed under anaerobic conditions in our previous study [17] is also operative in the presence of O_2. ˙NO itself is considered not to disproportionate [43]. However, several examples of ˙NO disproportionation promoted by transition metal complexes have been reported in Ref. [19]. To our knowledge, MnSOD-catalysed ˙NO dismutation is the first example of ˙NO disproportionation promoted by metallo-proteins that are widely present in biological systems. The molecular mechanism of MnSOD-catalysed ˙NO dismutation remains to be fully elucidated and may prove to be a difficult task.

In our previous study, we demonstrated that under anaerobic conditions both NO^+ and HNO/NO^- generated by MnSOD-catalysed dismutation reacted with MnSOD causing extensive modification and inactivation [17]. The results of the present study show that during sustained aerobic exposure to micromolar levels of ˙NO (a situation reflective of *in vivo* conditions) the reaction of MnSOD with ˙NO predominated forming NO^-, $ONOO^-$ and H_2O_2.

Our evidence for the generation of $ONOO^-$ is derived from: (i) its reaction with exogenously added probe (DHR); (ii) the formation of substantial amounts of nitrate; (iii) the formation of substantial amounts of H_2O_2; and (iv) the tyrosine residue nitration and oxidation of MnSOD itself. The results suggest that both $ONOO^-$ decomposition and $ONOO^-$-dependent tyrosine residue nitration and oxidation are enhanced by metal centre-mediated catalysis [15]. The addition of synthetic $ONOO^-$ caused a significant level of DHR oxidation compared to that when MnSOD was present. This is consistent with a previous finding that MnSOD catalyses decomposition of $ONOO^-$ [15]. Significantly lower amounts (approximately 10 times less) of $ONOO^-$ were estimated from DHR oxidation than expected from the amounts of nitrate (the decomposition product of $ONOO^-$) found in the reaction mixture (Figure 4). The significant nitration and oxidation of MnSOD tyrosine residues detected in ˙NO-treated MnSOD suggest that manganese in the MnSOD active center enhances the enzyme autonitration [15].

The formation of substantial amounts of H_2O_2 upon aerobic MnSOD exposure to ˙NO observed in the present study deserves additional discussion. The reaction of NO^- with O_2, that has been suggested to take place via the intermediate production of $O_2^{\cdot-}$ which is then converted into H_2O_2 in the presence of SOD [31] (reactions 4–6) has been questioned in subsequent studies [38]. In the cited studies, Angeli's salt was used, which at neutral pH releases HNO [38], whereas in the present study, we dealt specifically with NO^- species bound to Mn in the enzyme active site. This may provide an explanation for the observed discrepancies.

Therefore, we propose that the major source of $ONOO^-$ generated upon aerobic MnSOD exposure to ˙NO is the reaction of MnSOD-derived NO^- with O_2. The formation of substantial amounts of H_2O_2 in the reaction mixture opens up the posibility that $ONOO^-$ might be subsequently formed in a reaction of H_2O_2 with ˙NO and MnSOD, as well as in a reaction of H_2O_2 with MnSOD-derived NO^+ species (reactions 7–8) [44,45].

$$\dot{N}O + H_2O_2 + \text{MnSOD} \rightarrow ONOO^- + H^+ + \text{MnSOD} \quad (7)$$

$$NO^+ + H_2O_2 \rightarrow ONOO^- + H^+ \quad (8)$$

Although the tyrosine residues modified by ˙NO-treated MnSOD were not identified, we postulate that ˙NO-treatment causes nitration of the critical tyrosine residue at position 34. This residue is located at the vertex of the substrate funnel [2,46,47] suggesting that the tyrosine residue may come into contact with nitrating species generated upon reaction of $ONOO^-$ with Mn in the active site [15]. Consistent with findings reported for human recombinant MnSOD [13,42], we can predict the position where MnSOD (*E. coli*) homodimers cross-link: the covalent binding of two tyrosine 174 residues, which are located in close proximity on the surfaces of MnSOD (*E. coli*) subunits facilitating 3,3′-dityr formation via the combination of two tyrosyl radicals.

However, in contrast to MnSOD treatment with synthetic $ONOO^-$ which yields 3-HT and minimal levels of dityr [42], high yields of dityr relative to those of 3-nitrotzrosine were found in ˙NO-treated MnSOD (Figure 5A). This means that in ˙NO-treated MnSOD, the other pathways are operative in addition to $ONOO^-$-mediated dityr formation [13,42]. We assume that dityr could have been formed in a reaction of $TyrO^{\cdot}$ with ˙NO, which is known to yield dityr as a major product under the conditions such as those of our study in which the flux of ˙NO was higher than that of $O_2^{\cdot-}$ [48]. Besides this, we speculate that tyrosine residues could be oxidised by the strong oxidant of currently unknown structure, which may be

formed upon auto-oxidation of HNO [37,38] formed by protonation of MnSOD-derived NO^- (reaction 2).

MnSOD (*E. coli*) bears high structural and functional similarities to human MnSOD found in mitochondria [47]. Whereas the human mitochondrial enzyme is homotetramer [47], *E. coli* MnSOD is a dimer [46]. However, MnSOD from mitochondria has a metal site that is nearly indistinguishable from that of *E. coli* MnSOD [47]. The effect of synthetic $ONOO^-$ on *E. coli* MnSOD is quite similar to that reported for human recombinant MnSOD [15]. Taking these points together we assume that the $^{\cdot}NO$-mediated effects on *E. coli* MnSOD observed in our present study in which biologically relevant MnSOD ($20 \pm 10\,\mu M$ in mitochondria [49]) and $^{\cdot}NO$ concentrations ($1-10\,\mu M$ under pathological conditions [50]) were used may also be relevant for human MnSOD.

It is widely assumed that the major source of $ONOO^-$ *in vivo* is the extremely rapid reaction of $^{\cdot}NO$ with $O_2^{\cdot-}$ [16,39]. $ONOO^-$ is a strong oxidant and nitrating agent that promotes oxidative damage via a variety of mechanisms. Due to the multiplicity of its targets $ONOO^-$ has a short half-life suggesting that most of it will react/be consumed in close proximity to its site of production [39]. Although there can be no doubt that synthetic $ONOO^-$ effectively nitrates and oxidises proteins, the generation of $ONOO^-$ and $ONOO^-$-mediated protein nitration *in vivo* has been questioned in a number of recent studies especially when $^{\cdot}NO$ and $O_2^{\cdot-}$ are generated from independent sources [48]. A number of studies have pointed to MnSOD as a predominant nitrated protein [4–6,11,12], suggesting a high sensitivity of MnSOD towards nitration and/or its close proximity to the oxidant source. Concentrations of O_2 *in vivo* are many orders of magnitude higher than those of $O_2^{\cdot-}$ (between $5-200\,\mu M$ and $< 60\,pM$, respectively [51]) suggesting that although the reaction between NO^- and O_2 proceeds at a slower reaction rate than that of $^{\cdot}NO$ and $O_2^{\cdot-}$, the former mechanism of $ONOO^-$ formation could be potentially relevant to the explanation of MnSOD nitration and inactivation *in vivo*. MnSOD tyrosine nitration and oxidation by $ONOO^-$ generated at the enzyme active site would more convincingly explain the observed high sensitivity of MnSOD towards nitration *in vivo* under conditions of up-regulated $^{\cdot}NO$ synthesis, such as those occuring during ischemia/hypoxia [3–9], than the pathway based on MnSOD reaction with $ONOO^-$ generated from independent sources of $^{\cdot}NO$ and $O_2^{\cdot-}$.

Using a rodent model of renal ischemia/reperfusion, MacMillan and colleagues observed tyrosine nitration of MnSOD and cytochrome c during ischemia alone and demonstrated that these two proteins were specific targets of this oxidative damage [6]. Cytochrome c interacts with $^{\cdot}NO$, stimulates $^{\cdot}NO$ decay and catalyses the reduction of $^{\cdot}NO$ to NO^-, which subsequently reacts O_2 to yield $ONOO^-$ [29]. The capacity of MnSOD to stimulate $^{\cdot}NO$ decay as well as to induce $ONOO^-$ formation, reported in our study, is comparable or higher than that reported in the cited study for cytochrome c. Therefore, we interpret the above findings by assuming that interaction of these two proteins with $^{\cdot}NO$ (followed by its transformation into NO^-) preceeds and triggers the $ONOO^-$ formation via the reaction of NO^- with O_2, which then causes nitration and oxidation of specific tyrosine residues.

The proposed mechanism of MnSOD tyrosine nitration and inactivation may be applicable to other pathological situations associated with overproduction of $^{\cdot}NO$. Examples are MnSOD tyrosine nitration in the cerebrospinal fluid from patients suffering from neurodegenerative deseases [11] and in human lung adenocarcinoma cells in response to cytokines [12].

The results of our present study suggest that MnSOD may play a novel dual role in the formation and decomposition/scavenging of $ONOO^-$. MnSOD may catalyse $ONOO^-$ formation from $^{\cdot}NO$ facilitating both Mn-dependent $ONOO^-$ decomposition and $ONOO^-$-mediated tyrosine residue nitration [15]. This suggests that most of the generated $ONOO^-$ will be decomposed into nitrate and/or consumed in nitration and oxidation of MnSOD tyrosine residues. In this way, MnSOD may protect other cell constituents from $ONOO^-$-dependent oxidations. Dismutation of $O_2^{\cdot-}$ to H_2O_2 by MnSOD (reaction 5) reduces the formation of $ONOO^-$ (reaction 6), prolongs the half-life of $^{\cdot}NO$ and increases the concentration of cytosolic H_2O_2 level. H_2O_2 generated in this manner may then be involved in the regulation of different cellular processes [51] and/or contribute to oxidative cell damage [52].

A recent study has examined the role of enzymes as protectors against high local concentrations of $^{\cdot}NO$ in $^{\cdot}NO$ generating cells. It was proposed that these enzymes might regulate the level of $^{\cdot}NO$ via their reductase or dismutase activities [53]. The results presented in our previous report [17] together with the present study raise the possibility that MnSOD is a candidate for being a $^{\cdot}NO$ dismutase and points to the novel mechanism by which MnSOD may protect the cell from deleterious effects associated with overproduction of $^{\cdot}NO$. The mechanisms include reactions of GSH with both MnSOD-derived NO^+ and HNO/NO^- species which yield GSNO and hydroxylamine, respectively and protects the enzyme from $^{\cdot}NO$-mediated structural modifications and inactivation. However, $^{\cdot}NO$-mediated MnSOD inactivation could at the same time amplify the toxic effects of $^{\cdot}NO$.

On the basis of our *in vitro* study, we argue that the reactions of $^{\cdot}NO$ with *E. coli* MnSOD may represent part of the biochemical basis of resistance to $^{\cdot}NO$-mediated host defense [54]. Reactive $^{\cdot}NO$ species serving as part of host defense have DNA as their

ultimate target [46] and *E. coli* MnSOD is effective in preventing damage to DNA [55]. By reducing the levels of both $O_2^{\cdot-}$ and ˙NO, MnSOD decreases the extent to which $O_2^{\cdot-}$ combines with ˙NO to form $ONOO^-$ in the cell that is more toxic than either of its precursors.

The results presented herein and in our previous study [17] together with the finding that trace amounts of iron ions (reflective of the so-called "free iron" in biological milieu) may stimulate ˙NO dismutation into NO^+ and HNO/NO^- species [18] illustrate that metal-assisted ˙NO dismutation in biological systems may be feasible and possibly physiologically relevant. The relevance of these findings in the case of human MnSOD as well as the importance of metal-assisted ˙NO dismutation *in vivo* warrants further consideration along the lines presented here.

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References

[1] Michelson AM. Free radicals and disease: Tretment and clinical application with superoxide dismutase. In: Johnson JE, Walford R, Harmon D, Miguel J, editors. Free radicals, aging and degenerative diseases. New York: A.R. Liss Inc. 1986. p 263.

[2] Stroupe ME, DiDonato M, Tainer AJ. Manganese superoxide dismutase. In: Messerschmidt A, Huber R, Poulos T, Wieghardt K, editors. Handbook of metalloproteins. John Wiley & Sons: Chichester, NY; 2001. p 941–951.

[3] MacMillan-Crow LA, Cruthirds DL. Invited review: Manganese superoxide dismutase in disease. Free Radic Res 2001;34:325–336.

[4] MacMillan-Crow LA, Crow JP, Kerby JD, Beckman JS, Thompson JA. Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. Proc Natl Acad Sci USA 1996;93:11853–11858.

[5] MacMillan-Crow LA, Cruthirds DL, Ahki KM, Sanders PW, Thompson JA. Mitochondrial tyrosine nitration precedes chronic allograft nephropathy. Free Radic Biol Med 2001;31:1603–1608.

[6] Cruthirds DL, Novak L, Akhi KM, Sanders PW, Thompson JA, MacMillan-Crow LA. Mitochondrial targets of oxidative stress during renal ischemia/reperfusion. Arch Biochem Biophys 2003;412:27–33.

[7] Pittman KM, MacMillan-Crow LA, Peters BP, Allen JB. Nitration of manganese superoxide dismutase during ocular inflammation. Exp Eye Res 2002;74:463–471.

[8] Gray KD, MacMillan-Crow LA, Simovic MO, Stain SC, May AK. Pulmonary MnSOD is nitrated following hepatic ischemia-reperfusion. Surg Infect (Larchmt) 2004;5:66–173.

[9] Guo W, Adachi T, Matsui R, Xu S, Jiang B, Zou MH, Kirber M, Lieberthal W, Cohen RA. Quantitative assessment of tyrosine nitration of manganese superoxide dismutase in angiotensin II-infused rat kidney. Am J Physiol Heart Circ Physiol 2003;285:H1396–H1403.

[10] van der LB, Labugger R, Skepper JN, Bachschmid M, Kilo J, Powell JM, Palacios-Callender M, Erusalimsky JD, Quaschning T, Malinski T, Gygi D, Ullrich V, Luscher TF. Enhanced peroxynitrite formation is associated with vascular aging. J Exp Med 2000;192:1731–1744.

[11] Aoyama K, Matsubara K, Fujikawa Y, Nagahiro Y, Shimizu K, Umegae N, Hayase N, Shiono H, Kobayashi S. Nitration of manganese superoxide dismutase in cerebrospinal fluids is a marker for peroxynitrite-mediated oxidative stress in neurodegenerative diseases. Ann Neurol 2000;47:524–527.

[12] Aulak KS, Miyagi M, Yan L, West KA, Massillon D, Crabb JW, Stuehr DJ. Proteomic method identifies proteins nitrated *in vivo* during inflammatory challenge. Proc Natl Acad Sci USA 2001;98:12056–12061.

[13] MacMillan-Crow LA, Crow JP, Thompson JA. Peroxynitrite-mediated inactivation of manganese superoxide dismutase involves nitration and oxidation of critical tyrosine residues. Biochemistry 1998;37:1613–1622.

[14] Yamakura F, Taka H, Fujimura T, Murayama K. Inactivation of human manganese-superoxide dismutase by peroxynitrite is caused by exclusive nitration of tyrosine 34 to 3-nitrotyrosine. J Biol Chem 1998;273:14085–14089.

[15] Quijano C, Hernandez-Saavedra D, Castro L, McCord JM, Freeman BA, Radi R. Reaction of peroxynitrite with Mn-superoxide dismutase. Role of the metal center in decomposition kinetics and nitration. J Biol Chem 2001;276:11631–11638.

[16] Radi R, Peluffo G, Alvarez MN, Naviliat M, Cayota A. Unraveling peroxynitrite formation in biological systems. Free Radic Biol Med 2001;30:463–488.

[17] Niketic V, Stojanovic S, Nikolic A, Spasic M, Michelson AM. Exposure of Mn and FeSODs, but not Cu/ZnSOD, to NO leads to nitrosonium and nitroxyl ions generation which cause enzyme modification and inactivation: An *in vitro* study. Free Radic Biol Med 1999;27:992–996.

[18] Stojanovic S, Stanic D, Nikolic M, Spasic M, Niketic V. Iron catalyzed conversion of NO into nitrosonium ($NO+$) and nitroxyl ($HNO/NO-$) species. Nitric Oxide 2004;11:256–262.

[19] Ford PC, Lorkovic IM. Mechanistic aspects of the reactions of nitric oxide with transition-metal complexes. Chem Rev 2002;102:993–1018.

[20] Lewis RS, Deen WM. Kinetics of the reaction of nitric oxide with oxygen in aqueous solutions. Chem Res Toxicol 1994;7:568–574.

[21] Donald CE, Hughes MN, Thompson JM, Bonner FT. Photolysis of the N=N bond in trioxodinitrate: Reaction between triplet NO^- and O_2 to form peroxynitrite. Inorg Chem 1986;25:2676–2677.

[22] Wink DA, Feelisch M. Formation and detection of nitroxyl and nitrous oxide. In: Feelisch M, Stamler JS, editors. Methods in nitric oxide research. London: Wiley; 1996. p 403–412.

[23] Keele BB, Jr., McCord JM, Fridovich I. Superoxide dismutase from *escherichia coli* B. A new manganese-containing enzyme. J Biol Chem 1970;245:6176–6181.

[24] Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 1972;247:3170–3175.

[25] Beckman SJ, Wink AD, Crow PJ. Nitric oxide and peroxynitrite. In: Feelisch M, Stamler JS, editors. Methods

in nitric oxide research. London: John Wiley and sons; 1996. p 61–70.
[26] Malencik DA, Sprouse JF, Swanson CA, Anderson SR. Dityrosine: Preparation, isolation, and analysis. Anal Biochem 1996;242:202–213.
[27] Di Iorio E. Preparation of derivatives of ferrous and ferric hemoglobin. Methods Enzymol 1981;76:57–72.
[28] Lee M, Arosio P, Cozzi A, Chasteen ND. Identification of the EPR-active iron-nitrosyl complexes in mammalian ferritins. Biochemistry 1994;33:3679–3687.
[29] Sharpe MA, Cooper CE. Reactions of nitric oxide with mitochondrial cytochrome c: A novel mechanism for the formation of nitroxyl anion and peroxynitrite. Biochem J 1998;332:9–19.
[30] Stamler J, Feelisch M. Preparation and detection of S-nitrosothiols. In: Feelisch M, Stamler JS, editors. Methods in nitric oxide research. London: John Wiley and sons; 1996. p 521–540.
[31] Kirsch M, de Groot H. Formation of peroxynitrite from reaction of nitroxyl anion with molecular oxygen. J Biol Chem 2002;277:13379–13388.
[32] Ioannidis I, de Groot H. Cytotoxicity of nitric oxide in Fu5 rat hepatoma cells: Evidence for co-operative action with hydrogen peroxide. Biochem J 1993;296:341–345.
[33] Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Anal Biochem 1982;126:131–138.
[34] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–685.
[35] Hogg N, Singh RJ, Kalyanaraman B. The role of glutathione in the transport and catabolism of nitric oxide. FEBS Lett 1996;382:223–228.
[36] Shafirovich V, Lymar SV. Nitroxyl and its anion in aqueous solutions: Spin states, protic equilibria, and reactivities toward oxygen and nitric oxide. Proc Natl Acad Sci USA 2002;99:7340–7345.
[37] Miranda KM, Espey MG, Yamada K, Krishna M, Ludwick N, Kim S, Jourd'heuil D, Grisham MB, Feelisch M, Fukuto JM, Wink DA. Unique oxidative mechanisms for the reactive nitrogen oxide species, nitroxyl anion. J Biol Chem 2001;276:1720–1727.
[38] Miranda KM, Dutton AS, Ridnour LA, Foreman CA, Ford E, Paolocci N, Katori T, Tocchetti CG, Mancardi D, Thomas DD, Espey MG, Houk KN, Fukuto JM, Wink DA. Mechanism of aerobic decomposition of Angeli's salt (sodium trioxodinitrate) at physiological pH. J Am Chem Soc 2005;127:722–731.
[39] Hughes MN. Relationships between nitric oxide, nitroxyl ion, nitrosonium cation and peroxynitrite. Biochim Biophys Acta 1999;1411:263–272.
[40] Reif A, Zecca L, Riederer P, Feelisch M, Schmidt HH. Nitroxyl oxidizes NADPH in a superoxide dismutase inhibitable manner. Free Radic Biol Med 2001;30:803–808.
[41] van d V, Eiserich JP, O'Neill CA, Halliwell B, Cross CE. Tyrosine modification by reactive nitrogen species: A closer look. Arch Biochem Biophys 1995;319:341–349.
[42] MacMillan-Crow LA, Thompson JA. Tyrosine modifications and inactivation of active site manganese superoxide dismutase mutant (Y34F) by peroxynitrite. Arch Biochem Biophys 1999;366:82–88.
[43] Kanner J. Nitric oxide and metal-catalyzed reactions. Methods Enzymol 1996;269:218–229.
[44] McBride AG, Borutaite V, Brown GC. Superoxide dismutase and hydrogen peroxide cause rapid nitric oxide breakdown, peroxynitrite production and subsequent cell death. Biochim Biophys Acta 1999;1454:275–288.
[45] Kim YS, Han S. Nitric oxide protects Cu,Zn-superoxide dismutase from hydrogen peroxide-induced inactivation. FEBS Lett 2000;479:25–28.
[46] Edwards RA, Baker HM, Whittaker MM, Whittaker JW, Jameson GB, Baker EN. Crystal structure of *Escherichia coli* manganese superoxide dismutase at 2.1—ANG. resolution. J Biol Inorg Chem 1998;3:161–171.
[47] Borgstahl GE, Parge HE, Hickey MJ, Beyer WF, Jr., Hallewell RA, Tainer JA. The structure of human mitochondrial manganese superoxide dismutase reveals a novel tetrameric interface of two 4-helix bundles. Cell 1992;71:107–118.
[48] Goldstein S, Czapski G, Lind J, Merenyi G. Tyrosine nitration by simultaneous generation of (·)NO and O-(2) under physiological conditions. How the radicals do the job. J Biol Chem 2000;275:3031–3036.
[49] Tyler DD. Polarographic assay and intracellular distribution of superoxide dismutase in rat liver. Biochem J 1975; 147:493–504.
[50] Groves JT. Peroxynitrite: Reactive, invasive and enigmatic. Curr Opin Chem Biol 1999;3:226–235.
[51] Cadenas E. Mitochondrial free radical production and cell signaling. Mol Aspects Med 2004;25:17–26.
[52] Farias-Eisner R, Chaudhuri G, Aeberhard E, Fukuto JM. The chemistry and tumoricidal activity of nitric oxide/hydrogen peroxide and the implications to cell resistance/susceptibility. J Biol Chem 1996;271:6144–6151.
[53] Jay-Gerin JP, Ferradini C. Are there protective enzymatic pathways to regulate high local nitric oxide (NO) concentrations in cells under stress conditions? Biochimie 2000;82:161–166.
[54] Nathan C, Shiloh MU. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. Proc Natl Acad Sci USA 2000;97:8841–8848.
[55] Hopkin KA, Papazian MA, Steinman HM. Functional differences between manganese and iron superoxide dismutases in *Escherichia coli* K-12. J Biol Chem 1992;267:24253–24258.