Mechanism of p21^{Ras} S-Nitrosylation and Kinetics of Nitric Oxide-Mediated Guanine Nucleotide Exchange†

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ABSTRACT: Nitric oxide (NO), a highly reactive redox molecule, can react with protein thiols and protein metal centers to regulate a multitude of physiological processes. NO has been shown to promote guanine nucleotide exchange on the critical cellular signaling protein p21^{Ras} (Ras) by S-nitrosylation of a redox-active thiol group (Cys^{118}). This increases cellular Ras−GTP levels in vivo, leading to activation of downstream signaling pathways. Yet the process by which this occurs is not clear. Although several feasible mechanisms for protein S-nitrosylation with NO and NO donating have been proposed, results obtained from our studies suggest that Ras can be S-nitrosylated by direct reaction of Cys^{118} with nitrogen dioxide ($^{\bullet}NO_2$), a reaction product of NO with O_2, via a Ras thiyl-radical intermediate (Ras-S$^{\bullet}$). Results from our studies also indicate that Ras Cys^{118} can be S-nitrosylated by direct reaction of Cys^{118} with a glutathionyl radical (GS$^{\bullet}$), a reaction product derived from homolytic cleavage of S-nitrosoglutathione (GSNO). Moreover, we present evidence that reaction of GS$^{\bullet}$ with Ras generates a Ras-S$^{\bullet}$ intermediate during GSNO-mediated Ras S-nitrosylation. The Ras-S$^{\bullet}$ radical intermediate formed from reaction of the Ras thiol with either $^{\bullet}NO_2$ or GS$^{\bullet}$, in turn, reacts with NO to complete Ras S-nitrosylation. NO and GSNO modulate Ras activity by promoting guanine nucleotide dissociation from Ras. Our results suggest that formation of the Ras radical intermediate, Ras-S$^{\bullet}$, may perturb interactions between Ras and its guanine nucleotide substrate, resulting in enhancement of guanine nucleotide dissociation from Ras.

Nitric oxide (NO)[1] can react with a variety of cellular molecules to regulate a host of cellular processes including muscle relaxation, vasodilation, ion channel activity, and cellular growth control (*1*). Emerging interest in NO as a regulator of cell signaling molecules stems from recent data demonstrating that NO can modify cellular signaling targets (i.e., growth factor receptors, kinases, and phosphatases) and alter their activities (*2*). An important component of NO biochemistry involves the formation of protein S-nitrosothiol (PSNO) from a target protein thiol (PSH) in vivo (*3*). S-nitrosylation and transnitrosation reactions with PSH may represent an important signaling mechanism, because these reactions are believed to regulate the activity of many enzymes (*4*−*10*).

Among the many known proteins that are regulated by S-nitrosylation, the protooncogene protein product, p21^{Ras} (Ras), is of high interest, since Ras is known to be a focal point of many diverse cellular signaling pathways (*11*). We and others have shown that NO can regulate Ras activity by stimulating the slow intrinsic dissociation of guanine nucleotide substrates from Ras via S-nitrosylation of a solvent-accessible cysteine at position 118 (Cys^{118}). This results in enhanced guanine nucleotide exchange on Ras and activation of Ras in vivo (*12*−*17*).

It is well-known that S-nitrosylation of low-molecular-weight thiols (LMW RSHs) can occur in the presence of O_2 through a dinitrogen trioxide (N_2O_3) intermediate (Scheme 1A) (*18*−*21*). A radical-based S-nitrosylation mechanism has also been proposed in which nitrogen dioxide ($^{\bullet}NO_2$), a reaction product of NO with O_2, oxidizes LMW RSH to produce a low-molecular-weight thiyl radical intermediate (LMW RS$^{\bullet}$) (Scheme 1B) (*22*, *23*). Transient formation of the glutathionyl radical (GS$^{\bullet}$) has been observed upon incubation of a NO donor spermine NONOate with glutathione (GSH) (*24*) and further reaction of LMW RS$^{\bullet}$ with NO results in LMW RSH S-nitrosylation (Scheme 1B) (*22*). Another mechanism proposed to operate at physiological concentrations of NO ($<\sim 2\ \mu$M) under anaerobic conditions, is the direct reaction of NO with LMW RSH to produce a radical intermediate, putatively R−S−N$^{\bullet}$−O−H, which in turn is converted to low-molecular-weight S-nitrosothiol (LMW RSNO) by reduction of an electron acceptor (A, Scheme 1C) (*25*). Scheme 1E describes a distinct S-nitrosylation mechanism that involves conversion of NO

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[1] Abbreviations: carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DTH, sodium dithionite; GSH, glutathione; GSNO, *S*-nitrosoglutathione; Hb, hemoglobin; LMW RSH, low-molecular-weight thiol; LMW RSNO, low-molecular-weight nitrosothiol; NO, nitric oxide; PBN, phenyl *N*-*tert*-butylnitrone; PSNO, protein S-nitrosothiol; PSH, protein thiol; SHE, standard hydrogen electrode; spermine NONOate, (*Z*)-1-[*N*-(3-ammoniopropyl)-*N*-[4-(3-aminopropylammonio)butyl]-amino]diazen-1-ium-1,2-diolate.

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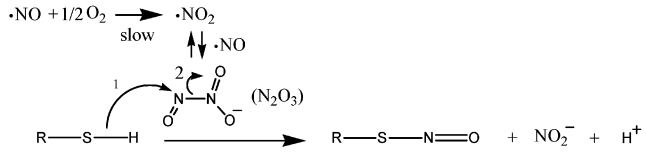
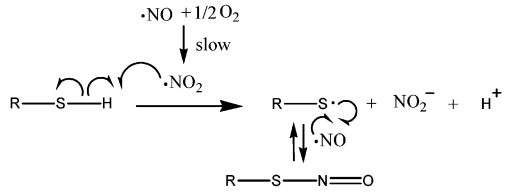
Scheme 1: Various Thiol S-Nitrosylation Reactions[a]

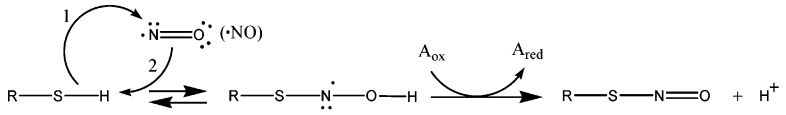
A. N_2O_3: a reaction product of NO radical with O_2



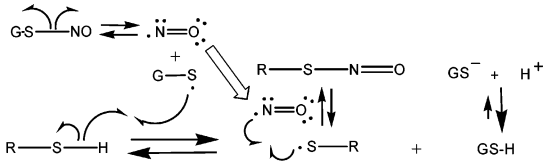
B. NO_2 radical: a reaction product of NO radical with O_2



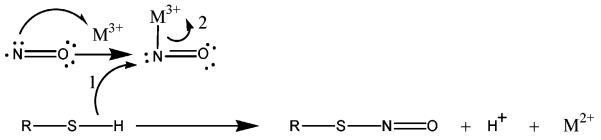
C. NO radical with an electron acceptor (A) under anaerobic condition



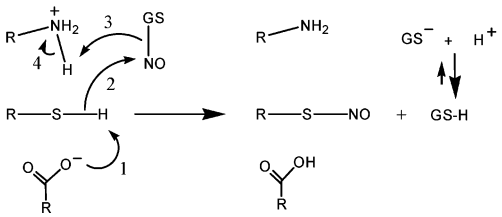
D. Glutathionyl radical (GS•) and NO radical released from the decomposition of GSNO under anaerobic condition



E. NO cation (nitrosonium ion) as a form of metal adduct



F. Acid-base mechanism



[a] The putative order of reaction processes is numbered in sequence.

radical to a nitrosonium ion (NO^+) by transition metal ions (M) (*26*).

NO produced from nitric oxide synthase (NOS) at a remote location may be delivered to a target PSH by conversion of NO to LMW RSNO or abundantly expressed S-nitrosoproteins (e.g., serum albumin) (*3*, *27*). Thus, mechanisms involving LMW RSNO-mediated S-nitrosylation of proteins have also been postulated (Scheme 1D). The basic mechanism of LMW RSNO-mediated S-nitrosylation (Scheme 1D) is similar to $^{\bullet}NO_2$-mediated S-nitrosylation of LMW RSH (Scheme 1B), except that the LMW RS• radical intermediate is formed by reaction of LMW RSH with GS• radical, a product of the homolytic cleavage of the GSNO S−N bond, rather than reaction of LMW RSH with $NO_2^{\bullet}$ (*26*, *28*, *29*). It is anticipated that the reaction mechanisms in Scheme 1A−D are applicable to PSH, unless the chemical environment of the PSH is altered significantly (i.e., solvent accessibility, pK_a, protein−protein interactions, or protein−ligand interactions).

In addition to these NO radical-based mechanisms, a distinct S-nitrosylation mechanism (Scheme 1F) for PSH has been proposed. This mechanism requires an acid−base pair in the protein for transfer of a NO group from LMW RSNO to a target PSH (Scheme 1F) (*4*, *30*, *31*). This acid−base mechanism may be particularly relevant to Ras S-nitrosylation, because the guanine nucleotide-binding motif (NKCD) of Ras contains an acid−base pair (D^{119} and K^{117}) near the residue in Ras (Cys^{118}) that is S-nitrosylated (*13*, *14*).

Although some of these reaction mechanisms have been characterized for LMW RSH, there is still uncertainty

regarding the mechanism of S-nitrosylation of PSH in vitro and in vivo. The present study defines a reaction path for Ras S-nitrosylation that is relevant to Ras activation in vitro and potentially in vivo.

MATERIALS AND METHODS

Preparations of Chemicals. The chemicals used for all experiments were of the highest grade unless otherwise documented. Both nitric oxide gas (NO, 98.5%) and nitrogen gas (N_2, 99.9%) were obtained from Aldrich. NO gas was purified by passing it through a scrubbing column with 5 M KOH. To optimize formation of S-nitrosylated Ras from N_2O_3 or $^{\bullet}NO_2$, a NO/O_2 mixture was prepared by mixing purified NO gas with ambient O_2 at a ratio of $3NO/1O_2$ (v/v). 2′-(or-3′)-*O*-(*N*-methylanthraniloyl)guanosine 5′-diphosphate (mant-GDP) was purchased from Molecular Probes. To prevent metal-mediated conversion of the NO radical into nitrosonium ion (NO^+), all buffers used for kinetic and biochemical assays were passed through a metal-chelating Bio-Rad Chelex-100 cation exchange column (*32*). The metal content of the metal-chelated buffer was determined by inductively coupled plasma mass spectrometry (ICP-MS) at the University of Georgia, and the buffer was found to contain no trace metals (in the picomolar range). All protein samples were dialyzed with the metal-free buffer prior to performing any of the assays. Vials for the assays were washed with 1 N HCl and thoroughly rinsed with distilled water, and the highest pure grade of $MgCl_2$ and NaCl were used to avoid transition metal contamination in the assay buffers.

Experimental Conditions. Spectroscopic and kinetic studies were conducted in a closed system filled with inert N_2 gas to prevent diffusion of treated NO or NO/O_2 gas, so as to retain a constant effective sample concentration of treated NO or NO/O_2 gas over the assay period. Sample-containing vials were sealed with serum stoppers, and a vacuum manifold system was employed to displace O_2 with N_2. Sample transfers were conducted using O_2-free syringes by flushing the syringes with N_2. A mixed buffer containing 10 mM of MES, MOPS, TrisHCl, and HEPES was used for kinetic studies at various pHs (5.9−8.0), unless otherwise noted.

Quantification of NO. Quantification was achieved using a hemoglobin (Hb)-coupled assay (*33*) modified to measure NO under anaerobic conditions (see Supporting Information).

Preparation of Ras Proteins. Human H-Ras (1−166) and Ras C118S were expressed and purified as described previously (*34*). The final purities of the proteins were >95% as determined by SDS−PAGE. The Ras protein concentration was determined by the Bradford method (*35*).

Quantification of S-Nitrosylated Compounds. An effective direct method, distinct from those previously used (*3*, *36*, *37*) was employed for quantification of PSNO. In this method, the absorption spectrum near 542 nm was monitored to quantify PSNO formation (see Supporting Information). The content of PSNO was also measured using the Saville assay (*36*) with the Saville reagents, sulfanilamide (3%), $HgCl_2$ (0.3%), and *N*-(1-naphthyl)ethylenediamine dihydrochloride (0.1%) in 0.5 N HCl, all prepared anaerobically.

The total quantity of S-nitrosylated product (L_T) can be expressed as follows (see Supporting Information for derivation):

$$L_T = \frac{k_2[NO]}{K_{NO} + [NO]} + {}^{nonspecific}K_{NO}[NO] \qquad (5)$$

Kinetic Measurements of Guanine Nucleotide Exchange on Ras. Ras was preloaded with the fluorescence mant-labeled guanine nucleotide, mant-GDP, as described previously (*38*). The dissociation of mant-GDP from Ras (*38*, *39*) was measured as a change in the fluorescence intensity over time using a LS50B Perkin-Elmer fluorimeter. A standard assay mixture for the fluorescence measurements consisted of 20 μM GDP, 50 mM NaCl, and 5 mM $MgCl_2$ at various pHs (5.9−8.0). Fluorescence from mant-GDP was not quenched at NO concentrations less than 10 μM. While minor fluorescence quenching was observed at GSNO, SNP, and DMPO concentrations higher than 10 mM, the concentrations of these NO donating agents did not exceed 10 mM in any of the fluorescence experiments.

Determination of Kinetic Constants. Apparent NO/O_2-mediated dissociation rates of guanine nucleotide ($^{app}k_{GN}$) were determined by fitting the data to a single-exponential decay. The apparent dissociation constant for mant-GDP from Ras by NO ($^{app}K_D$) in the presence of O_2 at a fixed pH was determined by fitting the plot of $^{app}k_{GN}$ values at various concentrations of NO.

RESULTS

Potential Mechanisms of Ras S-Nitrosylation. Various mechanisms have been proposed for S-nitrosylation of LMW RSH with the reaction mechanisms shown in Scheme 1. To determine which of these reaction mechanisms may be applicable to Ras S-nitrosylation, a series of studies were conducted using NO gas and various NO generating reagents under aerobic and anaerobic conditions. Results from our studies indicate that in the presence of O_2 nearly 2 μM of dissolved NO can effectively S-nitrosylate wild-type (wt) Ras (Figure 1A). Because a reaction mixture of NO and O_2 (NO/O_2) produces $^{\bullet}NO_2$ and N_2O_3, Ras S-nitrosylation by NO under aerobic conditions may follow one or both of the reaction paths described in Scheme 1A,B. Since only a small amount of S-nitrosylated Ras was observed upon incubation of Ras with NO under anaerobic conditions, we believe that the residual S-nitrosylated Ras occurs as a result of minor O_2 contamination (Scheme 1A,B) under our assay conditions, rather than direct reaction of NO radical with Ras in the absence of O_2. Therefore, our results provide little support for Scheme 1C, anaerobic NO-mediated S-nitrosylation. When the radical spin-trapping reagent PBN was added to assay solution containing the NO/O_2 reaction mixture, minimal Ras S-nitrosylation was observed (Figure 1A). PBN can react with NO radical to form a NO−PBN adduct (*40*) thereby scavenging the available radical NO and diminishing production of the S-nitrosylating agent(s) N_2O_3 or $^{\bullet}NO_2$ or both and thereby the end-product of the reaction, Ras S−NO. PBN may also inactivate $^{\bullet}NO_2$ and Ras-S$^{\bullet}$ radical species, by formation of a NO_2−PBN or Ras-S−PBN adduct to prevent Ras S-nitrosylation. Although it is not clear which radical species is quenched by treatment with PBN, the data suggest that aerobic NO-mediated Ras S-nitrosylation may follow a radical-based mechanism (Scheme 1B). However,

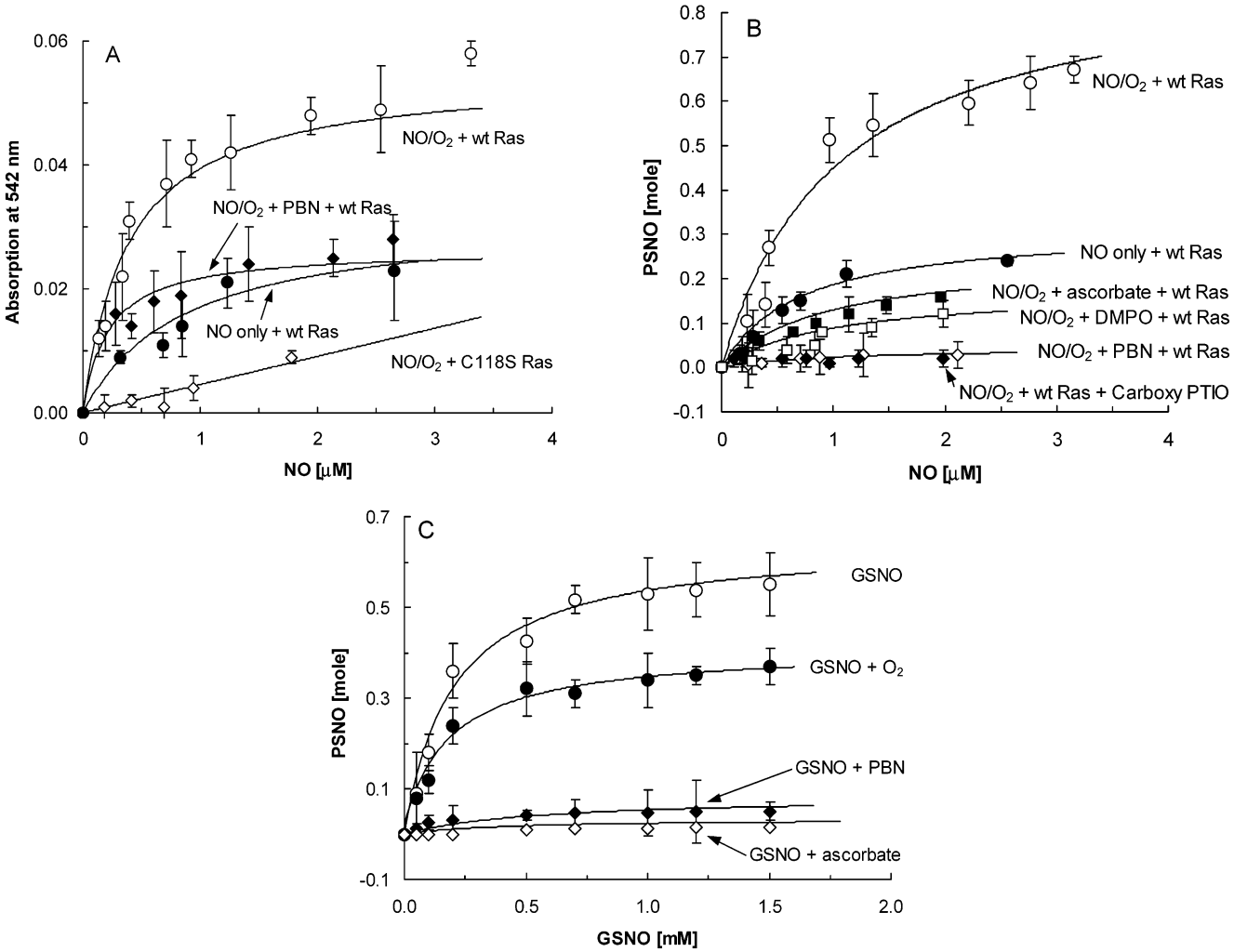


FIGURE 1: Quantification of Ras S-nitrosylation by NO, NO/O_2, and GSNO. In panel A, various amounts of NO gas were introduced into anaerobically sealed assay cuvettes containing a transition-metal-free buffer mixture (20 μM GDP, 5 mM $MgCl_2$, and 50 mM NaCl in 20 mM mixed buffer, pH 7.5). The quantity of NO adduct in the assay mixture was determined by using the Hb-coupled NO assay. For reaction of NO in the presence of O_2, the NO content in the assay mixture was determined by using the Hb-coupled NO assay prior to addition of stoichiometric amounts of O_2 ($3NO/1O_2$, v/v) to produce a NO/O_2 reaction mixture. The NO/O_2 reaction mixture was then incubated for 10 min. When indicated, the spin-trapping reagent, PBN (1 mM) and DMPO (10 mM), was added to either the NO- or NO/O_2-containing assay solution and incubated for 1 min prior to transfer of protein samples (wt or C118S Ras (0.5 μM)). The resulting absorption peak at 542 nm was monitored. In panel B, in addition to direct measurement of PSNO by monitoring the absorbance at 542 nm, the content of PSNO was also measured using the Saville assay (*36*). Experimental conditions and procedures were identical to those of panel A. In this assay, however, additional spin-trap reagents, carboxy-PTIO and ascorbate, were employed to trap radical species present in the assay mixtures. Carboxy-PTIO (0.1 mM) or ascorbate (1 mM) was added to assay buffer containing various concentrations of NO or the NO/O_2 mixture prior to adding the protein samples (0.5 μM). Saville reagent stocks were then added to the PSNO sample mixtures in the following order: (1) *N*-(1-naphthyl)ethylenediamine dihydrochloride, (2) $HgCl_2$, and (3) sulfanilamide (0.5 mL each). The mixture was incubated at room temperature for 10 min, and the absorbance was read at 540 nm. PSNO content was calculated according to a standard curve constructed with 0−5 μM $NaNO_2$, and the plot was fit to eq 5. In panel C, experimental conditions and data processing for GSNO-mediated Ras S-nitrosylation studies were identical to those in panel A, except that GSNO was used instead of NO and an arbitrary amount of ambient O_2 (100 μL) was added when indicated. GSNO-mediated S-nitrosylated wt Ras and unreacted GSNO have similar absorption intensity at 542 nm, so when GSNO was used, samples were applied to a size-exclusion column (Sephadex G-25) to remove unreacted GSNO. Gel filtration was performed within 2 min under anaerobic conditions. The absorption intensity at 542 nm was scaled by a dilution factor since gel filtration causes dilution of the sample. The dilution factors (1.5−1.9) for each individual protein sample were determined using the Bradford protein assay (*35*) after gel filtration. Absorbance at 542 nm was measured and peak intensity was plotted against various concentrations of NO. The plot was fit to eq 5. Values shown in panels A, B, and C represent mean values with standard errors obtained from measurements conducted in triplicate.

we cannot completely eliminate the nonradical N_2O_3-based S-nitrosylation mechanism (Scheme 1A), because N_2O_3 decomposes rapidly to produce NO radical and $^{\bullet}NO_2$ (*41*, *42*), which in turn may be sequestered by reaction with PBN, resulting in reduction of N_2O_3 in the assay mixture. Moreover, it is unlikely that nitrosonium ion (NO^+) serves as a NO donor for Ras S-nitrosylation (Scheme 1E), since transition metals (e.g., Cu^{2+} and Fe^{3+}) that facilitate formation of NO^+ from NO radical were eliminated from our assay system.

Because quantification of PSNO by monitoring the absorbance at 542 nm may be prone to significant error given its small extinction coefficient, the Saville assay (Figure 1B) was also employed to quantify PSNO formation by NO and the NO-donating agent GSNO. Results obtained from the Saville assay are consistent with absorbance measurements

at 542 nm and show that, while NO alone was not effective, a reaction mixture containing NO/O_2 effectively S-nitrosylates Ras. Moreover, by monitoring the absorption at 542 nm (Figure 1A), as well as the Saville assay (Figure 1B), formation S-nitrosylated Ras by NO/O_2 is blocked by PBN. Results shown in Figure 1B indicate that the NO radical spin-trapping reagent carboxy-PTIO (0.1 mM) (*43*) also effectively blocks NO/O_2-mediated Ras S-nitrosylation (Figure 1B). Addition of carboxy-PTIO most likely decreases the available NO radical, similar to PBN, and consequently reduces the level of active S-nitrosylating species in the NO/O_2 reaction mixture ($^{\bullet}NO_2$ and N_2O_3), minimizing formation of S-nitrosylated Ras. A third spin-trapping agent, ascorbate, also significantly diminishes NO/O_2-mediated Ras S-nitrosylation (Figure 1B). Notably, while ascorbate reacts with $^{\bullet}NO_2$ and thiyl radicals (i.e., glutathionyl radical, GS$^{\bullet}$) (*44*), it is not clear whether it reacts with NO radical. We propose that a Ras thiyl radical intermediate (Ras-S$^{\bullet}$) is formed by reaction with $^{\bullet}NO_2$, similar to the reaction mechanism described in Scheme 1B for LMW thiols. On the basis of this premise, inactivation of the putative Ras radical intermediate, Ras-S$^{\bullet}$, by ascorbate is likely to be more effective compared to the inactivation of $^{\bullet}NO_2$, since the reaction rate of ascorbate with thiyl radical is much faster (~30-fold) than that of ascorbate with $^{\bullet}NO_2$ (*23*, *45*). Results obtained from previous studies indicate that DMPO can trap GS$^{\bullet}$ (*24*). However, an excess amount of DMPO (at least 10 mM) was required compared to that of PBN and carboxy-PTIO to block Ras S-nitrosylation, indicating that DMPO may not be an effective Ras-S$^{\bullet}$ trapping agent under our experimental conditions (Figure 1B).

To determine the effective NO concentration required for NO/O_2-mediated Ras S-nitrosylation, the concentration dependence of NO/O_2-mediated S-nitrosylation of Ras was obtained (fit to eq 5) and the stoichiometry of S-nitrosylated Ras thiol determined. Approximately, 2 μM of dissolved NO was required to attain a unit absorption intensity of 0.005, which corresponds to Ras S-nitrosylation at a single PSH site (see Materials & Methods) (Figure 1A), and is consistent with the results obtained from the Saville assay (Figure 1B).

As shown in Figure 1C, S-nitrosylation of wt-Ras can also occur with GSNO as a NO-donor. Similar to NO/O_2-mediated Ras S-nitrosylation, GSNO-mediated S-nitrosylation of Ras was blocked by addition of the spin-trapping reagents, PBN (Figure 1C) and carboxy-PTIO (data not shown). As shown in Scheme 1D, GS$^{\bullet}$ and NO radical can be formed by the homolytic cleavage of GSNO (*46*). Despite the fact that PBN and carboxy-PTIO are known to react with NO (*40*, *43*), it is not known whether PBN and carboxy-PTIO react with GS$^{\bullet}$. Hence, while it is unclear whether PBN or carboxy-PTIO inactivate GS$^{\bullet}$, addition of PBN (and carboxy-PTIO) most likely inactivates the existing NO radical, thus depleting available NO radical, required to complete Ras S-nitrosylation (Scheme 1D), by formation of NO−PBN adduct. Moreover, ascorbate also effectively blocks GSNO-mediated Ras S-nitrosylation and may deactivate Ras-S$^{\bullet}$ (or GS$^{\bullet}$ or both), thereby preventing Ras S-nitrosylation.

GSNO was less effective in promoting Ras S-nitrosylation under aerobic conditions compared to anaerobic conditions (Figure 2). Since the GSNO homolytic cleavage product, GS$^{\bullet}$, can be oxidized to GS−OH in the presence of O_2 (*47*, *48*),

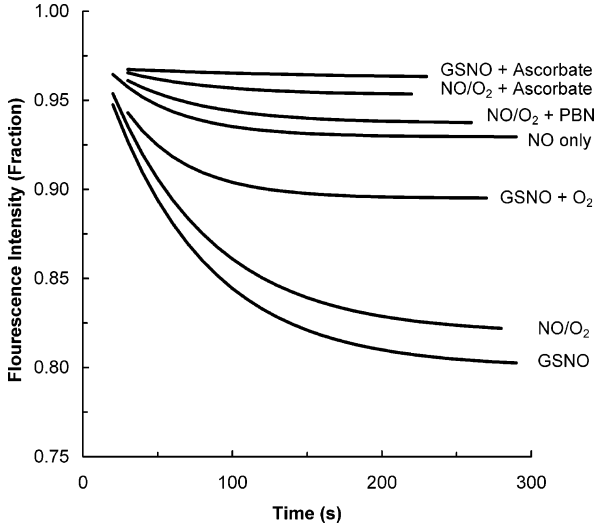


FIGURE 2: Kinetics of NO- and GSNO-mediated guanine nucleotide exchange on Ras in the presence and absence of O_2. NO (~2 μM) and GSNO (1 mM) were transferred into O_2-free sealed assay cuvettes containing assay buffer at pH 7.5, and NO content was determined prior to addition of any other reagents or protein sample. When indicated, a stoichiometric amount of O_2 ($3NO/1O_2$, v/v) and ambient O_2 (100 μL) was added into either the NO- or GSNO-containing assay solution respectively, and the spin-trapping reagent ascorbate (1 mM) or PBN (1 mM) was added and incubated for 1 min. Mant-GDP-loaded Ras (5 μM) was added, and the decrease in fluorescence emission at 460 nm was recorded as a function of time. Rates of mant-GDP dissociation from Ras were determined as described in Figure 2 and were 0.0001, 0.0002, 0.0007, 0.0012, 0.0038, 0.0041, and 0.0043 s^{-1} for GSNO with ascorbate, NO/O_2 mixture with ascorbate, NO/O_2 mixture with PBN, NO only, GSNO with O_2, NO/O_2 mixture, and GSNO, respectively.

O_2 may deplete the active radical species GS$^{\bullet}$ for Ras S-nitrosylation. Alternatively, it is possible that NO radical released from homolytic cleavage of GSNO further reacts with O_2 to produce higher oxides such as $^{\bullet}NO_2$ and N_2O_3 (Scheme 1A). The formed $^{\bullet}NO_2$ may generate more Ras-S$^{\bullet}$ (Scheme 1B). Hence, the presence of O_2 in the Ras GSNO mixture may either increase or decrease formation of Ras-S$^{\bullet}$. Although, it is unclear whether the presence O_2 decreases or increases the quantity of Ras-S$^{\bullet}$, we postulate that the presence of O_2 diminishes the quantity of Ras-S$^{\bullet}$ under our experimental conditions since O_2 reduces GSNO-mediated Ras S-nitrosylation.

The Site of NO-Mediated S-Nitrosylation on Ras. Cys^{118} has previously been identified as the site of Ras (1−166) S-nitrosylation under aerobic conditions (*13*, *14*). Since Cys^{118} is the only solvent-accessible PSH in Ras (1−166), it is a likely residue to be modified by NO gas. Consistent with these previous findings, a variant of Ras lacking a cysteine at position 118 (C118S Ras) shows negligible S-nitrosylation upon treatment with NO at concentrations <2 μM (Figure 1A). A comparative mass spectrometry analysis for the reaction products of wt Ras (1−166) and C118S Ras treated with NO at concentrations of ~2 μM shows that a single PSH site of wt Ras (1−166) is S-nitrosylated, whereas C118S Ras remains unmodified (data not presented). These results indicate that the primary radical NO-mediated S-nitrosylation target site in Ras (1−166) is Cys^{118}.

Kinetics of NO- and GSNO-Mediated Guanine Nucleotide Exchange on Ras. We have characterized Ras guanine nucleotide exchange kinetics under aerobic and anaerobic

conditions in the presence and absence of various NO-generating and spin-trapping reagents (Figure 2). While mant-GDP dissociation from Ras was enhanced in the presence of NO/O_2, with a rate of 0.0038 s^{-1}, minimal (0.0012 s^{-1}) NO-mediated mant-GDP dissociation was observed under anaerobic conditions. Figure 2 also shows that NO/O_2-mediated Ras guanine nucleotide dissociation was impeded by the spin-trapping reagent PBN, suggesting that NO/O_2 treatment generates a radical species that promotes guanine nucleotide exchange on Ras (Scheme 1B). Based on findings that ascorbate inactivates thiyl radical species and also inhibits NO/O_2-mediated Ras nucleotide dissociation (Figure 2), we postulate that Ras-S$^\bullet$ is the active radical species that facilitates NO/O_2-mediated guanine nucleotide exchange on Ras.

We have also investigated the reaction kinetics of Ras with GSNO, because it has been previously proposed that GSNO may act as a protein S-nitrosylation adduct in vivo. Similar to NO/O_2, we found that GSNO effectively promotes guanine nucleotide dissociation from Ras (Figure 2). Hence, it is possible that both NO/O_2 and GSNO (Scheme 1D) react with the Ras thiol to form a Ras-S$^\bullet$ intermediate that, in turn, promotes guanine nucleotide dissociation from Ras (Figure 2). We therefore anticipated that the thiyl radical spin-trapping agent ascorbate would impede GSNO-mediated Ras guanine nucleotide dissociation. Consistent with this premise, addition of ascorbate inhibits mant-GDP dissociation from Ras mediated by GSNO (Figure 2), providing further support that a Ras radical intermediate, Ras-S$^\bullet$, is the key activating species for GDP dissociation from Ras. The results also suggest that nonradical based mechanisms, such as the acid−base mechanism (Scheme 1F) do not account for the observed NO-mediated guanine nucleotide exchange on Ras.

Not too surprising based on our earlier results on Ras S-nitrosylation, when O_2 was present in a reaction mixture containing Ras and GSNO, Ras guanine nucleotide dissociation was inhibited (Figure 2), because the presence of O_2 is likely to deplete the thiyl radical species, GS$^\bullet$ or Ras-S$^\bullet$, thereby inhibiting NO- or GSNO-mediated guanine nucleotide dissociation from Ras.

Saturation kinetic studies of NO/O_2-mediated mant-GDP dissociation from Ras show that the intrinsic dissociation rate of mant-GDP from Ras (0.000 02 s^{-1}) was enhanced maximally 200-fold by NO gas at concentrations of 2 μM in the presence of O_2 (Figure 3). The apparent dissociation constant for mant-GDP from Ras by NO ($^{app}K_D$) in the presence of O_2 is estimated to be 1.7 ± 0.4 μM [NO] (Figure 3). These kinetic results are compatible with a stoichiometric amount of NO in the presence of O_2 (~2 μM, see Figure 1A,B) that is required for effective S-nitrosylation of a single PSH. Minor S-nitrosylation at additional Ras PSH site(s) at NO concentrations >2 μM was observed in the presence of O_2 but was not associated with additional changes in NO/O_2-mediated guanine nucleotide dissociation from Ras. Stimulation of mant-GDP dissociation from C118S Ras by treatment with NO (<2 μM) was not observed (data not presented). NO concentrations less than 2 μM were unable to S-nitrosylate C118S Ras whereas a single PSH of wt Ras was effectively S-nitrosylated at NO concentrations of 2 μM (Figure 1A,B). Taken together, these findings indicate that enhanced GDP dissociation from Ras is coupled with the S-nitrosylation process at a single site, Cys^{118}.

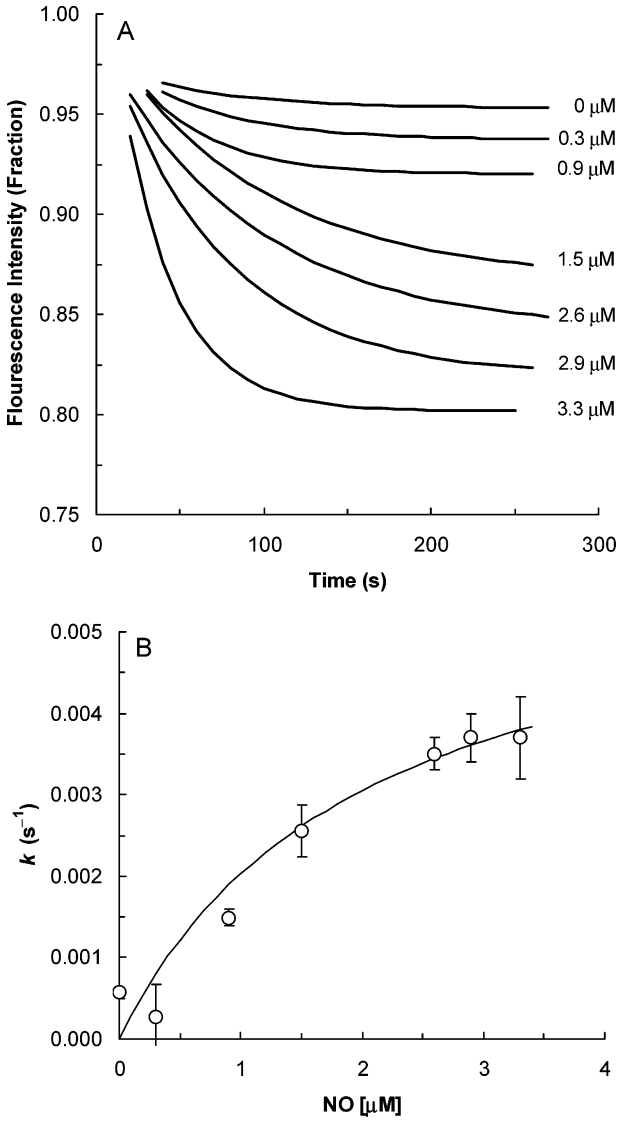


FIGURE 3: Ras mant-GDP guanine nucleotide dissociation in the presence of NO/O_2. In panel A, various amounts of the NO/O_2 reaction mixture were introduced into standard assay buffer. NO/O_2-mediated Ras guanine nucleotide dissociation was initiated by addition of mant-GDP-loaded Ras (5 μM). The decrease in fluorescence emission at 460 nm was monitored over a time period ranging from 0 to 300 s, and the data were fit to a simple exponential decay to determine apparent NO/O_2-mediated enhanced rates of mant-GDP dissociation from Ras ($^{app}k_{GN}$). In panel B, the estimated $^{app}k_{GN}$ values at various concentrations of the NO/O_2 reaction mixture were plotted against the concentration of NO. The plot was fit to a hyperbola, giving 1.7 ± 0.4 μM [NO] (R^2 = 0.8995), an apparent dissociation constant for NO-mediated dissociation of mant-GDP from Ras in the presence of O_2 ($^{app}K_D$). Concentrations of the NO/O_2 reaction mixture in panels A and B were expressed as the NO concentration determined by the Hb-coupled assay, as described in Supporting Information.

Characterization of the Chemical Species Involved in NO-Mediated Guanine Nucleotide Exchange on Ras. The site of Ras(1−166) S-nitrosylation, Cys^{118}, lies in a highly conserved motif (NKXD where X is Cys^{118}). Hence, it is possible that the process of Cys^{118} nitrosylation perturbs nearby interactions between conserved residues within the NKXD motif of Ras and its bound guanine nucleotide substrate. X-ray crystal and NMR solution structures combined with mutagenesis data of residues in the NKXD motif that form interactions with the guanine nucleotide base

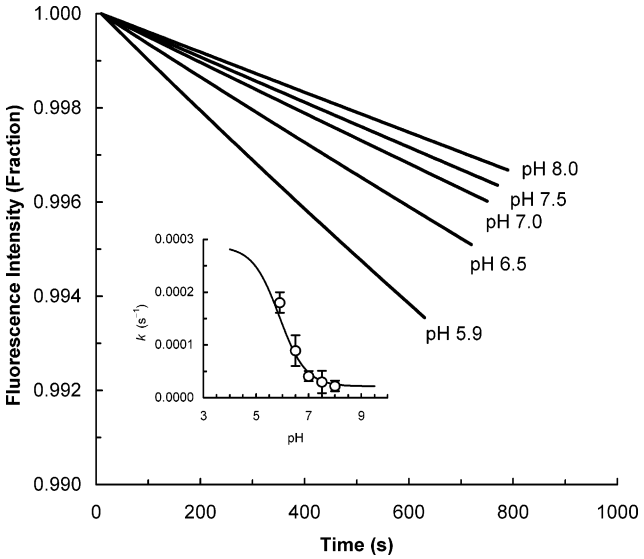


FIGURE 4: pH-dependent intrinsic guanine nucleotide dissociation rates from Ras. Mant-GDP-loaded Ras (5 μM) was placed in O_2-free sealed cuvettes containing assay buffer (pH 5.9−8.0). The decrease in the fluorescence emission at 460 nm due to dissociation of fluorescent nucleotide was followed with time, and the rates were fit to single-exponential decay. In the inset, the rates of nucleotide exchange at various pHs were determined and plotted against pH, and the plot was fit to the monoprotic Henderson−Hasselbalch equation (*53*).

indicate that a web of hydrogen bonds between Ras and either GDP or GTP is critical for high-affinity and specific interaction of guanine nucleotide substrates with Ras (Supporting Information, Scheme 1) (*49*−*52*). Therefore, one of the products of Ras S-nitrosylation common to all reactions in Scheme 1, the proton (H^+), may promote perturbation of guanine nucleotide binding to Ras. To determine whether changes in local H^+ concentration may perturb hydrogen bond interactions between Ras and GDP, we examined Ras guanine nucleotide dissociation rates as a function of pH. We found that guanine nucleotide dissociation from Ras is sensitive to pH in the pH range from 5.9 to 8.0 (Figure 4). An apparent monoprotic pK_a (pK_{app}) value of 5.9 ± 2.3 (Figure 4 inset) was determined by fitting the data to the Henderson−Hasselbalch equation (*53*). The pK_{app} may correspond to a weighted sum of the pK_a's of various ionization groups in the NKCD motif of Ras or represent NKCD motif-independent ionization(s), which could directly or indirectly affect guanine nucleotide binding to Ras. Further studies are required to resolve this issue. Nevertheless, quantitative analysis of guanine nucleotide dissociation from Ras at different pHs suggests that maximally a 15-fold stimulation of guanine nucleotide dissociation from Ras is observed by changing 2 pH units (from pH 8.0 to pH 5.9). The magnitude of stimulation is not as great as that mediated by a minimal amount of NO in the presence of O_2, where ~2 μM of NO/O_2 enhances the dissociation rate of the guanine nucleotide at least 200-fold. This suggests that H^+ minimally perturbs guanine nucleotide binding on Ras. Other factor(s), such as production of a radical intermediate Ras-S$^\bullet$ (vide infra), may have a more significant role in destabilizing the binding of the guanine nucleotide substrate on Ras.

DISCUSSION

In this study, we have evaluated six distinct mechanisms of Ras S-nitrosylation to assess the means by which NO mediates guanine nucleotide exchange on Ras in the presence of O_2. Multiple factors were evaluated to relate the chemistry of NO reaction with LMW RSH to Ras S-nitrosylation under intracellular conditions. Our results suggest that direct reaction of Ras Cys^{118} with either a NO/O_2 reaction product, $^\bullet NO_2$, or GSNO reaction product, GS$^\bullet$, generates a Ras radical intermediate state, (postulated to be Ras-S$^\bullet$), which can react with NO radical to produce S-nitrosylated Ras. Ras S-nitrosylation most likely proceeds via Scheme 1, reaction B or D, with either a NO/O_2 adduct or NO-releasing agent (i.e., GSNO) serving as a NO donor, respectively.

It is unlikely that the reaction process shown in Scheme 1F promotes Ras S-nitrosylation since the functional groups associated with the $NKC^{118}D$ motif, vicinal to the nitrosylated thiol of Ras (where K/D corresponds to the acid/base motif in Ras), are tightly coupled to the guanine nucleotide substrate through multiple binding interactions (*49*−*52*) (Supporting Information, Scheme 1). Moreover, a variant of Ras containing a D119N substitution can still be S-nitrosylated under similar conditions to those used for S-nitrosylation of wild-type Ras (see Supporting Information, Figure 3), suggesting that the base−cysteine−acid motif in Ras is not critical for S-nitrosylation. Furthermore, our findings indicate that Ras S-nitrosylation by NO gas and NO donating agents under both anaerobic and aerobic conditions is a radical-based process whereas the mechanism described in Scheme 1F is a non-radical-based reaction.

As shown in Scheme 1E (*26*, *54*), transition metals (Cu^{2+} and Fe^{3+}) catalyze oxidation of NO radical to NO^+. However, for our experiments, transition metals were removed through the use of metal-free buffers and acid-washed vials. Hence, it is unlikely that NO^+ is the reaction species that mediates aerobic Ras S-nitrosylation by NO, because treatment of Ras with NO/O_2 under metal-free assay conditions yields S-nitrosylated Ras (Figure 1A,B).

To understand the complete cycle of aerobic NO-mediated Ras activation, the chemistry of the reverse process, denitrosylation of S-nitrosylated Ras, should also be considered. It is likely that Ras denitrosylation proceeds through homolytic cleavage of the S−N bond between NO and the thiol of Cys^{118}, since homolytic cleavage of the S−N bond, the radical mechanism in reverse, is a dominant process for denitrosylation of S-nitroso compounds (i.e., GSNO and SNP) (*26*, *28*, *29*).

The kinetic and chemical results presented herein suggest that the reaction process of Cys^{118} nitrosylation promotes guanine nucleotide dissociation from Ras. In particular, our data indicate that a radical intermediate (conceivably Ras-S$^\bullet$) produced by a reaction of $^\bullet NO_2$ (a reaction product of NO with O_2) or LMW RSNO with Ras promotes dissociation of guanine nucleotide from Ras. Mant-GDP dissociation from Ras was not stimulated by either $^\bullet NO_2$ or GS$^\bullet$ when Ras thiol residues were chemically modified by *N*-methylmaleimide (NEM, data not shown), indicating enhanced guanine nucleotide exchange by NO does not result from direct interaction of either $^\bullet NO_2$ or GS$^\bullet$ with Ras. The intrinsic guanine nucleotide dissociation rate in NEM-treated Ras was similar to that of non-S-nitrosylated Ras (0.000 12 s^{-1}), indicating

that chemical modification does not alter the kinetics of Ras guanine nucleotide exchange. Two byproducts of the reaction mechanisms shown in Scheme 1 are NO_2^- and H^+. Addition of NO_2^- (1−10 mM) to the Ras•GDP complex did not enhance guanine nucleotide dissociation (data not presented), whereas an increase in H^+ concentration (pH 8.0 to 5.9) was found to modestly enhance Ras guanine nucleotide dissociation. It is therefore possible that the H^+ byproduct also plays a role in NO-mediated Ras guanine nucleotide dissociation.

The molecular basis for Ras radical-mediated perturbation of guanine nucleotide binding is not clear at this time. Additional studies are required to delineate how S-nitrosylation of Ras leads to perturbation of GDP binding both in vivo and in vitro. Activation of Ras by other redox agents (i.e., O_2^- and Hg) has been proposed on the basis of in vitro studies (*12*). It will be of interest to determine how other redox agents, in addition to NO, may lead to activation of Ras in vivo.

Our studies were performed with truncated Ras (1−166), which contains only one redox-sensitive site, Cys^{118}, to generate Ras-S•. However, full-length H-Ras contains additional carboxyl-terminal redox-sensitive cysteine residues (Cys^{181}, Cys^{184}, and Cys^{186}). These cysteine residues are sites of posttranslational lipid modification, including both farnesylation and palmitoylation (*55*). Posttranslational lipid modification at these sites is important for plasma membrane targeting and biological activity of Ras. A recent study has shown that addition of 4 mM S-nitrosocysteine (CysNO) to NIH 3T3 cells promotes enhanced turnover of Ras palmitoylation, presumably due to NO-modification of Cys^{181} and Cys^{184} in Ras (*56*). The enhanced turnover of Ras palmitoylation did not affect membrane localization of Ras but was believed to result in Ras deactivation and attenuation of MAP kinase signaling. These studies were conducted under open atmosphere. Given results from our present study, it is possible that reaction of the C-terminal Ras Cys^{181} and Cys^{184} thiols with cysteinyl radical (Cys-S•) generated from the homolytic cleavage of CysNO, could promote S-nitrosylation at Cys^{181} and Cys^{184} (as well as Cys^{118}) for the following reasons. First, it has been shown that homolytic cleavage of CysNO produces Cys-S• and NO radical (*46*). Second, it has been reported that the fraction of NO produced from millimolar amounts of CysNO is in the micromolar regime (*46*). As shown in Scheme 1D, it is anticipated that a 1:1 stoichiometric amount of NO and Cys-S• can be produced from homolytic cleavage of CysNO. This indicates that Cys-S• produced from millimolar amounts of CysNO would be in the micromolar regime. Third, the thiyl radical species, Cys-S• or Ras-S•, can be oxidized to produce inactive thiol species in the presence of O_2. It is also possible that NO radical released from homolytic cleavage of CysNO further reacts with O_2 to produce a higher oxide such as $•NO_2$, which may generate Ras-S• by reaction with the Ras thiol. When O_2 is present in the Ras GSNO mixture, Ras guanine nucleotide dissociation was decreased, suggesting that O_2 reduces the Ras-S• content (see Results section). Given these results, in conjunction with the structural similarity between GSNO and CysNO (*46*), we anticipate that aerobic preparation of CysNO could also cause diminution of Ras-S•. Hence, because the studies performed by Baker et al (2000) were conducted under open atmosphere in the presence of O_2, the amount of Ras-S• is likely to be greatly reduced compared to our strict anaerobic conditions. Thus, CysNO-mediated S-nitrosylation of Cys^{181} and Cys^{184} under aerobic conditions most likely requires an excess amount of CysNO (4 mM) (*56*) for S-nitrosylation to occur.

Although treatment of cells with 4 mM CysNO may lead to nitrosylation of Cys^{181} and Cys^{184} in full-length Ras, the physiological significance of this study is unclear (*56*). First, CysNO-mediated Ras palmitoylation turnover was observed only in NIH 3T3 cells but not in PC12 cells. Second, other nitrosylation agents, some of which have previously been demonstrated to modify Cys^{118} of Ras and lead to Ras activation, had no effect on Ras palmitoylation. Third, it has become increasing clear that the conditions employed and concentrations of exogenously supplied nitrosylation agents used for in vitro and in vivo studies should be investigated carefully. For example, we have observed a decrease in Ras activity at higher concentrations of nitrosylating agents due to modification of sites on Ras other than surface accessible cysteines (data not shown). Consistent with our observations, a decrease in Ras activity or Ras-mediated signaling has been observed at higher concentrations of nitrosylation agents in a previous study, where the dose dependence of various nitrosylating agents has been investigated (*57*). Finally, no evidence for endogenous NO-mediated enhanced palmitoylation turnover of Ras has been observed in vivo. Hence, it is difficult to ascertain whether nitrosylation of Ras Cys^{181} and Cys^{184} occurs and results in increased turnover of Ras palmitoyl sites under conditions of endogenous NO generation. However, if altered Ras palmitoylation does occur under conditions where Ras is also modified on Cys^{118}, the effect on Ras may be complicated by a combination of Ras activation/deactivation events. Ras activation may occur due to enhanced guanine nucleotide exchange caused by modification of ^{118}Cys. However, if the Ras C-terminal cysteines are also modified, down regulation of Ras activity and Ras-mediated down-stream signaling events may result from modulation of Ras palmitoylation. The ultimate result, that is, whether Ras is activated or deactivated, may be dependent on the exact cellular conditions under study. Previous studies have demonstrated that truncated forms of Ras (1−166, 1−171) shows similar activity to the full-length protein (*58*). Consistent with these observations, we have observed that NO/O_2-mediated enhancement of guanine nucleotide dissociation in truncated Ras is similar to that observed in full-length Ras (data not shown). Since modification of C-terminal cysteine residues in full-length Ras does not affect Ras activity in vitro, we have focused our in vitro studies herein on a truncated form of Ras that lacks the C-terminal lipid modification sites, because we are unable to assess effects of lipid modification in vitro.

Although previous studies have demonstrated a role for NO in regulation of Ras guanine nucleotide exchange in vitro and Ras activation in vivo, neither quantitative nor mechanistic studies were conducted to assess the efficiency and mechanism of NO-mediated guanine nucleotide exchange on Ras. Results from this study suggest that a reaction product of NO with O_2, $•NO_2$ radical, as well as a homolytic cleavage product of GSNO, GS• radical, mediates Ras S-nitrosylation and enhances guanine nucleotide exchange via a Ras radical intermediate postulated to be Ras-S•. The radical mechanism may, therefore, serve as a novel and efficient mechanism for Ras activation in vivo.

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SUPPORTING INFORMATION AVAILABLE

Additional materials and methods information, expanded discussions of peripheral findings, and a supporting scheme and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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