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Redox Regulation of Ras and Rho GTPases: Mechanism and Function

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Abstract

Significance: Oxidation and reduction events are critical to physiological and pathological processes and are highly regulated. Herein, we present evidence for the role of Ras and Rho GTPases in controlling these events and the unique underlying mechanisms. Evidence for redox regulation of Ras GTPases that contain a redox-sensitive cysteine (X) in the conserved NKXD motif is presented, and a growing consensus supports regulation by a thiyl radical-mediated oxidation mechanism. We also discuss the debate within the literature regarding whether $2e^-$ oxidation mechanisms also regulate Ras GTPase activity. ***Recent Advances:*** We examine the increasing *in vitro* and cell-based data supporting oxidant-mediated activation of Rho GTPases that contain a redox-sensitive cysteine at the end of the conserved phosphoryl-binding loop (p-loop) motif (GXXXXG[S/T]C). While this motif is distinct from Ras, these data suggest a similar $1e^-$ oxidation-mediated activation mechanism. ***Critical Issues:*** We also review the data showing that the unique p-loop placement of the redox-sensitive cysteine in Rho GTPases supports activation by $2e^-$ cysteine oxidation. Finally, we examine the role that Ras and Rho GTPases play in controlling key oxidant-regulating enzymes in the cell, and we speculate on a feedback mechanism. ***Future Directions:*** Given that these GTPases and redox-regulating enzymes are involved in multiple physiological and pathological processes, we discuss future experiments that may clarify the interplay between them. *Antioxid. Redox Signal.* 18, 250–258.

Introduction

Oxidation and reduction reactions are important in numerous signaling pathways that regulate critical physiological processes, and the balance between oxidation and reduction reactions (redox state) is a critical contributor to many diseases. The reactive intermediates (RIs) involved in these processes include reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive thiols. Moreover, protein-RI reactions are diverse and can generate many products. While a number of review articles have concentrated on the diverse RI sources and elimination mechanisms found in cells, this review will focus on the regulation of select Ras and Rho proteins by reversible thiol oxidation.

Ras and Rho GTPases are involved in many fundamental signaling pathways that affect cell cycle, growth, apoptosis, motility, and morphology (25). Dysregulation of these proteins can lead to numerous disease states, including cancer, cardiovascular disease, and neurological disorders. Ras and Rho GTPases are molecular "switches." GTP binding alters their conformation, which promotes effector binding and turns downstream signaling "on," whereas the GDP-bound form reduces effector binding, turning signaling "off." In general, three distinct classes of proteins regulate the level of activated GTPases in the cell (Fig. 1).

Herein, we describe redox mechanisms involved in regulating Ras and Rho GTPases. We discuss how RIs alter the biochemical properties of these GTPases and the consequences on cell function. For Ras GTPases, recent publications that demonstrate a direct role for RI regulation of Ras in cancer pathogenesis are described. We also discuss evidence for indirect and direct RI regulation of Rho GTPases, highlight cell-based studies that show a strong role for Rho GTPases in regulating key redox-modulating enzymes, and propose a feedback mechanism for redox status in the cell. We conclude by suggesting areas for future research and experimental approaches to address remaining questions.

Redox Regulation of Ras and Rho GTPases: *In Vitro* Studies

Select free radical oxidants ($1e^-$) can regulate the activity of Ras superfamily GTPases, which possess key cysteine thiol(s). In these GTPases, $NO^{\bullet}/O_2$ and $^{\bullet}NO_2$ have been shown to induce guanine nucleotide oxidation and dissociation (21). As

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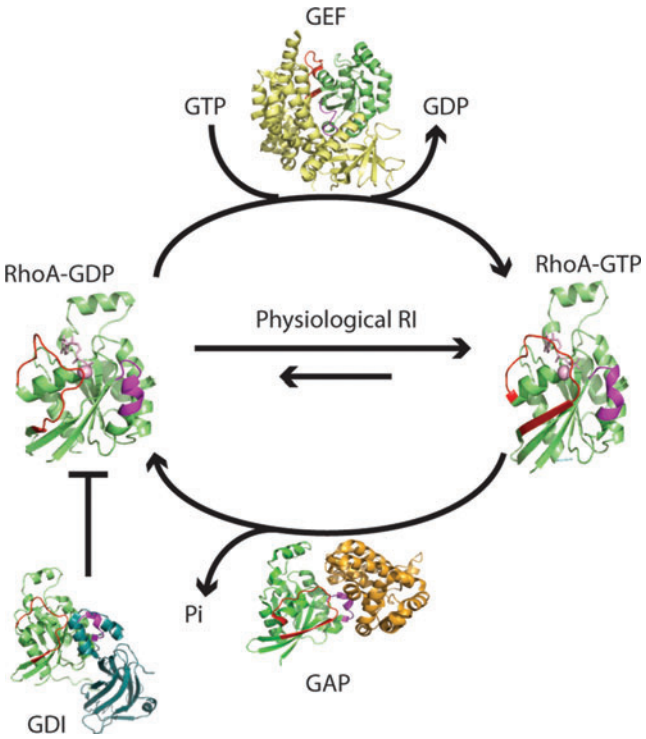

FIG. 1. GTPase nucleotide cycle. GTPases are active in the GTP-bound form. GTPase activating proteins (GAPs) promote GTP hydrolysis, which results in the inactive, GDP-bound form. Guanine nucleotide exchange factors (GEFs) facilitate nucleotide dissociation, and, as the in-cell GTP:GDP ratio is typically high (≥10:1), GTPases bind GTP by mass action and become activated. GDIs can also regulate Rho GTPases by sequestering them, which prevents nucleotide exchange and membrane insertion. We hypothesize that physiological levels of RIs facilitate exchange, which typically populates GTPases in the active conformation, whereas oxidative stress conditions inactivate the GTPases. RhoA is shown in green. The regions that undergo nucleotide-dependent changes in conformation are highlighted in red (switch I) and magenta (switch II). GEF is indicated in yellow, GAP is colored orange, and GDI is in teal. The following structures (PDB ID) were used to generate this model: RhoA-GTP (1A2B), RhoA-GTP with a GAP (1OW3), RhoA-GDP (1FTN), RhoA-GDP complexed with GDI (1CC0), and RhoA-GDP complexed with a GEF (1LB1). GDP, guanosine diphosphate; GDI, GDP dissociation inhibitor; RIs, reactive intermediates. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars.)

the intracellular GTP:GDP ratio is greater than or equal to 10:1 (50), redox-mediated nucleotide dissociation likely populates these GTPases in the active GTP-bound state; however, inactivation has also been observed, often correlated with oxidative stress. While there is still much debate on whether Ras GTPases are regulated by $2e^-$ (covalent) oxidation, a subset of Rho GTPases are activated by $1e^-$ and $2e^-$ cysteine oxidation. As RIs modulate the activity of several GTPases (Ras and Rho subclasses) involved in cell growth and motility, elucidating the mechanisms for GTPase regulation by redox control may provide new directions for drug targeting.

RI function, regulation, and production

RIs can be beneficial and harmful in biology. They comprise a number of reactive molecules that can interconvert into other RIs and control physiological processes by interacting with cellular components, including lipids, organelles, proteins, and nucleic acids (43, 54). Further, to maintain redox homeostasis in the cell and counterbalance the deleterious effects from RIs, cells have evolved a system of antioxidant molecules and enzymes. Excess RIs can cause changes in the cell redox potential, resulting in various disease states, such as atherosclerosis, cancer, diabetes, and neurodegenerative disorders. As previous reviews have detailed the chemistry underlying these reactions (4, 10, 53), this review will focus on RI-mediated cysteine oxidation and the effects on Ras and Rho GTPases.

ROS are produced *in vivo* by many enzymes and processes; examples include hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$), and hydroxyl radical ($^{\bullet}OH$). ROS can convert into other ROS forms through enzymes, metal catalysis, and free-radical-mediated reactions; for example, in the presence of transition metals, such as Cu^{2+} and Fe^{3+}, the Fenton reaction converts H_2O_2 into $^{\bullet}OH$, a short-lived and highly reactive free radical (55). NADPH oxidase (Nox) is a major source of $O_2^{\bullet -}$ in cells, and $O_2^{\bullet -}$ is converted into H_2O_2 by superoxide dismutase (SOD) (6, 36). Nitric oxide ($NO^{\bullet}$) is an important RNS in the cell and is produced by inorganic nitrate reduction and nitric oxide synthase (NOS) (38). Nitric oxide modulates multiple cell processes, such as blood pressure regulation, platelet aggregation, and smooth muscle relaxation (14). The effects of $NO^{\bullet}$ in vascular disease and cancer biology have been previously reviewed (32, 52). While the redox potential of nitric oxide is considered too low to directly generate thiyl-radical intermediates from protein thiols, its autooxidation product $^{\bullet}NO_2$ can (4). Peroxynitrite ($ONOO^-$) is another RNS and can be produced from the direct combination of $NO^{\bullet}$ with $O_2^{\bullet -}$ (5). Herein, we discuss mechanisms through which cellular NO generation leads to Ras and Rho GTPase activation.

Free radicals modify and activate Ras through a reactive cysteine in the NKCD motif

Ras superfamily GTPases have four conserved nucleotide-binding motifs (29). While the X residue in the NKXD motif is not well-conserved, several Ras subclass members contain a redox-active cysteine at this position (Supplementary Table S1). GTPases with a redox-sensitive NKCD motif can be activated by $^{\bullet}NO_2$ and other RIs; however, NO-mediated regulation is best characterized for Ras. Several cell-based and *in vitro* studies have shown that $^{\bullet}NO_2$ reacts with Ras through Cys^{118} to promote nucleotide exchange and Ras activation (10). Our lab has employed NMR and biochemical approaches to show that S-nitrosation of Ras does not affect Ras structure or nucleotide binding (56). We speculated that an intermediate formed during the reaction of Ras with $^{\bullet}NO_2$ modulates Ras activity and investigated several reactions involved in thiol S-nitrosation. Further, we observed that $1e^-$ oxidation (radical-mediated) reactions, which have been postulated to promote thiyl radical formation, enhance nucleotide dissociation from Ras (18). In support of these observations, we have recently confirmed Cys^{118} radical formation upon exposure of Ras to diethylamine NONOate ($NO^{\bullet}$-generating agent) and $^{\bullet}NO_2$, using immune-spin trapping and Fourier transform ion cyclotron resonance mass spectrometry (MS) (article in submission). As guanine bases are susceptible to

radical-mediated oxidation, we speculated that thiyl radical generation promoted oxidation of the Ras-bound guanine nucleotide. Supporting this premise, we used MS to demonstrate that 5-guanidino-4-nitroimidazole diphosphate (NIm-DP) is formed in the presence of $NO^{\bullet}/O_2$ and $^{\bullet}NO_2$. NIm-DP is a reported breakdown product of 5-nitro-guanine ribose diphosphate, which can be generated upon reaction of guanosine diphosphate with $^{\bullet}NO_2$ (21). Further, mutation of the redox-sensitive cysteine (Ras^{C118S}) prevented NO-mediated nucleotide oxidation and dissociation (20). These results suggest that $NO^{\bullet}/O_2$ or $^{\bullet}NO_2$ can produce a Ras thiyl radical ($Ras^{C118\bullet}$) that propagates to the guanine base by electron transfer (Fig. 2A). Guanine base oxidation disrupts critical intermolecular contacts between the nucleotide and Ras nucleotide binding pocket, which enhances nucleotide dissociation. These data suggest that Ras^{C118} thiol nitrosation does not promote nucleotide exchange; rather, exchange is promoted by thiyl radical formation at Ras^{C118} (Fig. 2A, B). Further supporting this model, we demonstrated that nucleotide binding is not altered by either Ras^{C118} mutation or S-nitrosation (20). A proposed thiyl-radical-based mechanism has been described in detail elsewhere (17) but remains inconclusive, as direct evidence to support the proposed radical intermediates has not yet been obtained.

In contrast to our observations that Cys^{118} nitrosation does not alter Ras activity, Clavreul *et al.* suggested that covalent modification of Ras by glutathione enhances nucleotide exchange (8). In these experiments, Ras^{WT}, but not Ras^{C118S}, was activated by glutathiolation using peroxynitrite, and it was hypothesized that Ras^{C118} glutathiolation by $2e^-$ oxidation (Fig. 2B) perturbs nucleotide binding because glutathione is negatively charged and larger than nitric oxide. Clavreul *et al.* concluded that glutathiolation significantly altered Ras structure at the nucleotide binding pocket and enhanced nucleotide exchange (8, 9). However, peroxynitrite was used in these experiments, and its breakdown products can generate RIs capable of thiyl radical formation. Peroxynitrite can undergo homolysis to generate $^{\bullet}NO_2$ and $^{\bullet}OH$. Moreover, in the presence of CO_2, peroxynitrite can produce carbonate radicals (39). While Kissner and Koppenol have observed that "radical-free" peroxynitrite homolysis produces nitrite and molecular oxygen (28), Szabo *et al.* discussed peroxynitrite homolysis products and concluded that it generates radical products (49). Therefore, it is impossible to judge whether the effects of glutathiolation on Ras activity were induced by free radicals or direct Cys^{118} modification. While Adachi *et al.* suggested that Ras S-nitrosation may be an intermediate during glutathione modification of Ras, they concluded that covalent ($2e^-$) glutathione modification of Ras leads to Ras activation (1). However, the observation that Ras glutathiolation alters nucleotide exchange contradicts our previous studies, which demonstrated that Cys^{118} and $1e^-$ oxidants are required for regulation of Ras nucleotide binding and activity. To address this discrepancy, we have recently demonstrated that treatment of Ras with oxidized glutathione (GSSG) results in glutathiolation of Ras at Cys^{118}. However, similar to S-nitrosation, glutathione modification of Ras does not alter Ras nucleotide binding (unpublished observations). Therefore, we

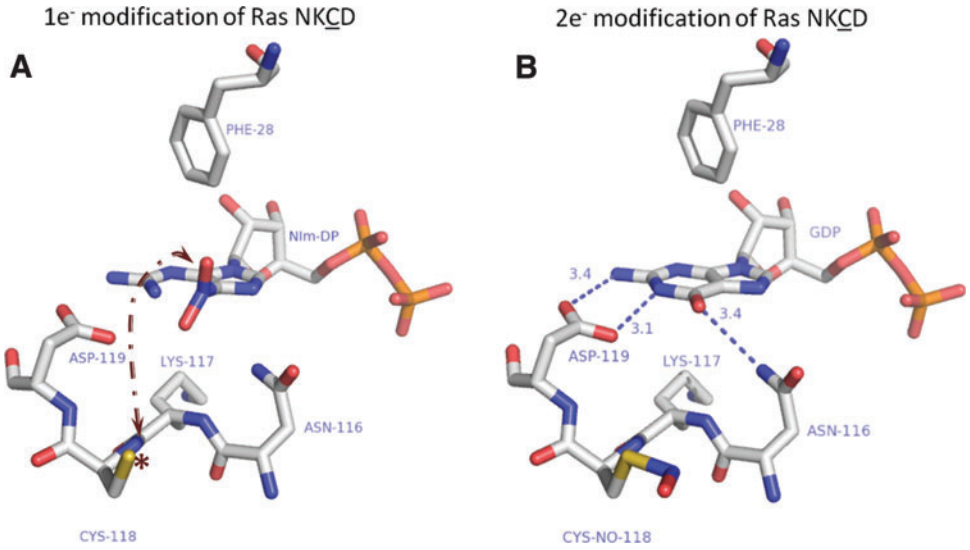

FIG. 2. Regulation of Ras guanine nucleotide binding by $1e^-$ and $2e^-$ oxidation. (A) Reaction of Ras with $^{\bullet}NO_2$ (a $1e^-$ oxidant) has been shown to generate NIm-DP, a breakdown product of 5-nitro-GDP. In the proposed mechanism, a thiyl radical is generated at Cys^{118} (*asterisk*) and transferred to the guanine base bound to Ras (*arrow*). Phe^{28} likely maintains the nucleotide orientation for proper radical transfer; however, the exact mechanism of electron transfer is under investigation. The end result of the radial-mediated reaction is oxidation of the guanine base, a loss of interactions between Ras and GDP (depicted by the absence of hydrogen bonds), and dissociation of the bound nucleotide. **(B)** A $2e^-$ (covalent) oxidation reaction of Ras with CysNO, which results in covalent modification of Cys^{118} with NO, does not affect the interactions with the bound nucleotide (hydrogen bonds are shown as *dotted blue lines*). Williams *et al.* showed using NMR that nitrosation of Cys^{118}, using CysNO, does not perturb the effector and switch regions of Ras (56). This observation was further reinforced by biochemical data that did not show a change in activity after covalent modification. Figure adapted from PDB ID:1CRP using Pymol. The color scheme is white, carbon; blue, nitrogen; salmon, oxygen; orange, phosphate; and yellow, sulfur. All distances are reported in angstroms. 5-nitro-GDP, 5-nitro-guanine ribose diphosphate; NIm-DP, 5-guanidino-4-nitroimidazole diphosphate. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars.)

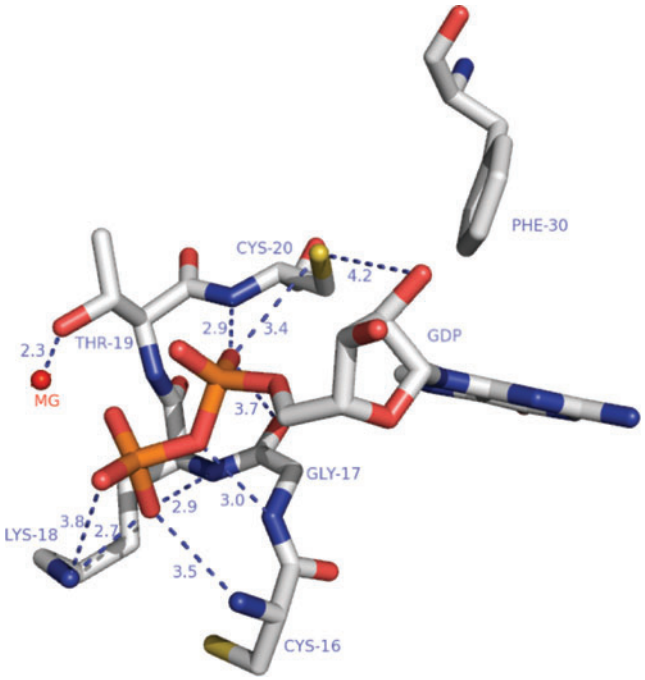

FIG. 3. The phosphoryl-binding loop of RhoA bound to GDP. The phosphoryl-binding loop of Rho GTPases, including Cys^{20} and Cys^{16}, makes several critical hydrogen bonds with the bound nucleotide. Oxidation of RhoA by $1e^-$ mediated oxidants results in nucleotide hydrolysis and dissociation similar to Ras GTPases; however, oxidation of $RhoA^{C20}$ by $2e^-$ oxidants likely results in perturbation of nucleotide binding and an increase in nucleotide exchange. According to data using phenyl-arsine oxide and peroxide (22), a mixed disulfide or disulfide can form between RhoA Cys^{20} and Cys^{16} upon oxidation of Cys^{20}. When the disulfide bond is formed, it occludes nucleotide binding and inactivates the GTPase. However, nucleotide binding can be restored upon reduction of the disulfide, and this cycling results in guanine nucleotide exchange (GDP for GTP) and activation of the GTPase. Figure adapted from PDB ID:1FTM using Pymol. The hydrogen bonds are shown as *blue dotted lines* with the distances in angstroms. Color scheme: carbon, white; sulfur, yellow; nitrogen, blue; oxygen, salmon; phosphate, orange; and magnesium, red. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars.)

propose that Ras activation requires Cys^{118} thiyl-radical formation for nucleotide dissociation and subsequent activation. One end product of Ras^{C118} thiyl-radical generation is Ras^{SSG}. Thus, the presence of Ras^{SSG} may reflect Ras activation if it was generated by a reaction with the Ras^{C118} thiyl radical; this is also the most likely mechanism in cells given the slow reaction rate for the Ras thiol and GSSG under physiological conditions (Fig. 2).

Redox regulation of Rho GTPases by $1e^-$ and $2e^-$ mechanisms through a redox-active cysteine in the phosphoryl-binding loop

Our lab has also demonstrated that select Rho GTPases are redox sensitive; however, Rho GTPases contain a different redox motif than that of Ras (19). Over 50% of Rho GTPases contain a redox-sensitive cysteine at the end of the nucleotide-binding phosphoryl-binding loop (p-loop) motif, GXXXXGK[S/T]C (Supplementary Table S2). Based on the Rac1, Cdc42, and RhoA crystal structures, the solvent-exposed cysteine in the p-loop motif likely has an altered pKa and is accessible to RIs. We have previously shown that the reactive p-loop cysteines in Rho GTPases are sensitive to oxidation, resulting in altered GTPase regulation (19).

Whereas treatment of RhoA with $^{\bullet}NO_2$ can promote nucleotide oxidation and dissociation similar to Rac1 and Cdc42, it can inhibit nucleotide binding in the absence of reducing agents. Distinct from Rac1 and Cdc42, RhoA contains two cysteines in its p-loop (Cys^{16} and Cys^{20}, Fig. 3; Supplementary Table S1). Exposure of RhoA with $^{\bullet}NO_2$ can promote disulfide bond formation between Cys^{16} and Cys^{20}, which occludes nucleotide binding (22). However, nucleotide binding can be restored with disulfide reducing agents. Our findings indicate that thiyl radical formation at $RhoA^{C20}$ upon $^{\bullet}NO_2$ treatment facilitates nucleotide oxidation and release, which increases $RhoA^{C16}$ accessibility and promotes intramolecular disulfide bond formation with Cys^{20}. RhoA disulfide bond formation occludes nucleotide binding and promotes RhoA inactivation. Based on these observations, we postulate that RhoA is likely activated by thiyl radical-promoting RIs under conditions where the redox potential is capable of promoting disulfide reduction and restoration of RhoA nucleotide binding (2, 44). Redox-mediated inactivation of Ras may occur when the cellular reduction potential is reduced, such as during oxidative stress. It is intriguing to speculate that enzymes, such as thioredoxin and glutaredoxin, which are capable of reducing protein disulfides, may act on RhoA and contribute to activation.

The addition of cisplatin or arsenic trioxide has been shown to inactivate RhoA by promoting mixed disulfide formation between the two p-loop cysteines (22). In contrast to Ras, these results suggest that $2e^-$ oxidation can regulate RhoA activity. In support of this hypothesis, Gerhard *et al.* showed that phenylarsine oxide generates a mixed disulfide crosslink between RhoA Cys^{16} and Cys^{20} using matrix-assisted laser desorption/ionization-MS and demonstrated that the modification inhibits stress fiber formation through the inactivation of RhoA in Caco-2 cells (15).

H_2O_2 has also been shown to regulate RhoA activity *in vitro* and in cells (2, 19). Unlike Ras^{C118}, $RhoA^{C20}$ directly interacts with the bound nucleotide (Fig. 4), and $2e^-$ oxidation of this cysteine likely perturbs nucleotide binding. We observed that $2e^-$ oxidation by peroxide at the p-loop cysteines can enhance the rate of nucleotide dissociation by ~10-fold. In addition, $1e^-$ oxidation has also been shown to induce nucleotide dissociation. Our lab has shown that $1e^-$ oxidants ($O_2^{\bullet -}$, $^{\bullet}NO_2$, and $^{\bullet}OH$) enhance RhoA nucleotide exchange by nearly 500-fold (19).

Conclusions

While work from our lab indicates that only $1e^-$ oxidation reactions that generate a thiyl radical at Cys^{118} in Ras affect Ras activity, discrepancies remain regarding $2e^-$ oxidation. Given the distinct Rho GTPase redox motif, $1e^-$ and $2e^-$ mechanisms can modulate their activity. RhoA is unique because $1e^-$ oxidants can induce a disulfide bond between Cys^{16} and Cys^{20}, which can inactivate RhoA by occluding nucleotide binding. While the reduction potential under most physiological conditions will likely promote disulfide bond

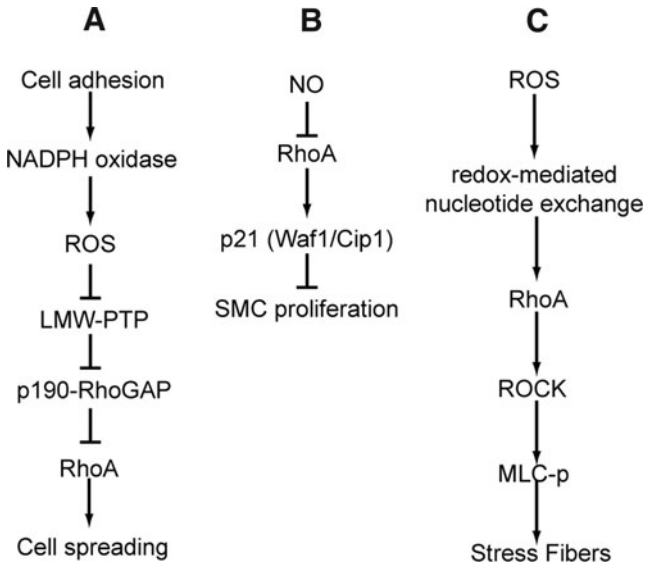

FIG. 4. RhoA signaling pathways. (A) Indirect inhibition of RhoA by Rac1-mediated ROS production. Cell adhesion and resulting Rac1 activation leads to ROS generation through Nox. ROS production inactivates LMW-PTP, resulting in increased phosphorylation of p190 Rho-GAP and, subsequently, its activation. Enhanced GAP activity results in decreased RhoA activity, which promotes cell spreading. **(B)** Inactivation of RhoA by NO leads to increased p21 expression. p21 expression inhibits smooth muscle cell proliferation. NO is thought to inactivate RhoA directly by S-nitrosation. **(C)** In addition to indirect regulation by RI, RhoA can be directly activated by ROS or RNS. ROS treatment of cells leads to enhanced nucleotide exchange and RhoA activation. RhoA activation enhances stress fiber formation. LMW-PTP, low-molecular-weight protein tyrosine phosphatase; MLC-p, phosphorylated myosin light-chain; Nox, NADPH oxidase; RNS, reactive nitrogen species; ROCK, Rho-associated protein kinase; ROS, reactive oxygen species; SMC, smooth muscle cells.

reduction and RhoA activation, under conditions of oxidative stress, radical quenching and/or disulfide reduction may not occur, which could render RhoA inactive (44). Further cell-based studies are needed to clarify the mechanisms of Rho GTPase regulation by redox agents.

Redox Regulation of GTPases: Cell-Based Studies

As we have recently published a detailed review on RNS regulation of Ras activity and biology (10), we will only highlight the most recent and salient data herein. Lander *et al.* first observed NO-mediated Ras activation in T cells (34), which was transient and concentration dependent. Later, it was shown that treatment with sodium nitroprusside, a nitric-oxide-generating compound, increased Ras downstream signaling through the mitogen-activated kinase pathway (33). An important tool in such studies, Ras^{C118S} is a variant that aids in determining the direct and indirect effects of RIs on Ras because it is RI insensitive and does not alter Ras structure and activity (41). A recent study on macrophages demonstrated that Ras S-nitrosation was required for Erk1/2-mediated apoptosis using the $NO^{\bullet}$ donor S-nitrosoglutathione (51). The Ras^{C118S} variant reversed the pro-apoptotic effects, indicating that the effects on cell survival were mediated by direct reaction with Ras. In this study, Tsujita *et al.* suggested that the process of nitrosation, rather than covalent modification, activated Ras (51). Our lab extended these observations and showed that Ras activation depends on $1e^{-}$ (radical)–mediated oxidation, which induces nucleotide dissociation (18). As most of these earlier studies used exogenous $NO^{\bullet}$ and/or were conducted in cells that overexpressed Ras, it was unclear whether $NO^{\bullet}$ regulated Ras activity under physiological conditions.

However, recent evidence has linked endothelial NOS (eNOS) to Ras nitrosation and activation in Ras-mediated tumorigenesis initiation and maintenance in mice. In the proposed activation pathway, oncogenic Ras activates the phosphoinositide 3-kinases (PI3K) pathway, which results in Akt-mediated (also protein kinase B) eNOS phosphorylation. Through a feedback mechanism, eNOS-generated $NO^{\bullet}$ activates Ras^{WT} and stimulates Ras downstream pathways (35). Further, Lim *et al.* showed that, for H- and N-Ras, introducing the Ras^{C118S} mutation circumvented PI3K pathway activation and reduced tumor growth. In a separate cell-based study, eNOS-derived $NO^{\bullet}$ activated N-Ras in T cells engaged with antigen presenting cells; Cys^{118} was required for activation (24). These data suggest that $NO^{\bullet}$ mediates Ras activation directly through Cys^{118}.

In-cell redox regulation of Rho GTPases

Nimnual *et al.* first described a pathway where ROS production was postulated to alter Rho GTPase activity by modulating Rho regulatory proteins (Fig. 4) (42). Decreased RhoA activity was observed in HeLa cells overexpressing constitutively active Rac1 ($Rac1^{CA}$). This effect was abolished when Rac1 was expressed without its "insert" region. The insert region is likely required for Nox activation and ROS production. $Rac1^{CA}$ depression of RhoA activity was inhibited when cells were treated with the ROS scavenger N-acetylcysteine; conversely, RhoA activity was inhibited when cells were exogenously treated with 1 m*M* H_2O_2. Moreover, enhanced p190RhoGAP tyrosine phosphorylation was observed with downstream Rac1 activation, and tyrosine phosphorylation was abolished in cells pretreated with an Nox inhibitor (diphenylene iodium) as well as cells expressing $Rac1^{CA}$ without the insert region. The authors further showed that activation of Rac1 inactivates a phosphatase (low-molecular-weight protein tyrosine phosphatase [LMW-PTP]), which may be upstream of p190RhoGAP. However, interpretation of these results may be complicated by the use of peroxide and N-acetylcysteine at high concentrations, which likely altered the cell redox state, redox targets, and signaling pathways. Further, their hypothesis that Rac1 drives ROS production through Nox to affect RhoA relies on the $Rac1^{CA}$ insert-deletion mutant (42). The importance of the insert region in Nox activation has been debated, and at least two reports describe the insert region as dispensable to ROS production (27, 40). However, Nox and Rac isoforms are expressed in different cell types and bind with varying efficiencies, which may account for this discrepancy, and downstream effects from $Rac1^{CA}$ may play a role. An alternative approach to insert-deletion mutants is necessary to interpret the mechanism for ROS regulation of RhoA.

Zuckerbraun *et al.* observed inactivation of RhoA by $NO^{\bullet}$ in smooth muscle cells after treatment with a pharmacological

$NO^{\bullet}$ donor (propylamine propylamine-NONOate) by measuring GTP and effector binding (Fig. 4) (57). Nitric oxide addition to smooth muscle cells decreased stress fiber formation, a phenotype often associated with RhoA inactivation. Further, exogenous $NO^{\bullet}$ enhanced ERK activity, upregulated the cyclin-dependent kinase inhibitor (p21), and decreased smooth muscle cell proliferation. Using $RhoA^{CA}$, the authors showed that $NO^{\bullet}$ inhibits smooth muscle cell proliferation through RhoA activation-dependent p21 upregulation. It was also shown that $NO^{\bullet}$ S-nitrosates RhoA when using NO release and nitrosocysteine immunodetection techniques. In the presence of the $NO^{\bullet}$ donor, GTP association was significantly inhibited, which was reversible with dithiothreitol (DTT). However, DTT reversal does not necessarily imply that S-nitrosation is the RhoA modification post-NO treatment because $NO^{\bullet}/O_2$ can yield a variety of different modifications, many reversible with DTT. An additional missing piece of key data is RhoA S-nitrosation site specificity. Based on work from Gerhard *et al.* (15) and our lab (22), Cys^{16} and Cys^{20} likely form a disulfide, and we hypothesize that Cys^{20} is the primary site of S-nitrosation.

We have recently shown that exogenous and endogenous ROS can stimulate RhoA activity (Fig. 4). In fibroblasts, RhoA activation and stress fiber formation were reversible by washing out peroxide and abolished upon N-acetylcysteine treatment. Moreover, we showed that ROS activation of RhoA was abolished when two critical cysteines were mutated ($RhoA^{C16A/C20A}$). Notably, $RhoA^{C16A/C20A}$ responds normally to guanine nucleotide exchange factor (GEF)–mediated activation and C3 toxin inactivation (2). These results show that ROS can directly activate RhoA through a mechanism involving Cys^{20} and/or Cys^{16}. These results differ from Nimnual *et al.*, as we observed direct RhoA activation by ROS (Fig. 4). Moreover, we identified the critical cysteines in ROS-mediated RhoA activation (22). Other observations have indicated that RhoA is activated in ROS-exposed cells, although a direct mechanism was not identified. As we have observed RhoA activation by ROS in fibroblasts, we hypothesize that high RI levels or a low cellular reduction potential can inactivate RhoA due to the irreversible formation of an RI-mediated RhoA Cys^{16}/Cys^{20} disulfide bond. Thus, our results do not implicitly disagree with Zuckerbraun *et al.* (57). Likely, RI activation and inactivation of GTPases depends on the RI levels and cell redox state. Low RI levels under high-reducing potential conditions may activate RhoA by promoting disulfide reduction and nucleotide exchange, whereas elevated RIs under low reducing potential conditions may inactivate RhoA (44).

Rho GTPases control redox-regulating enzymes

Rho GTPases play a key role in regulating cell redox status; they can modulate cell redox status in response to molecular and mechanical forces as well as an altered redox state. This response is particular to cell type, subcellular location, and initiating event; thus, the downstream effects are diverse and highly regulated. In cell-based and animal experiments, Rac proteins (Rac1 and Rac2 isoforms) and RhoA regulate expression and activity of primary redox-modulating enzymes in the cell: Nox ($O_2^{\bullet -}$ generation), NOS ($NO^{\bullet}$ generation), and SOD ($O_2^{\bullet -}$ dismutation into H_2O_2) (Fig. 6). As RIs can modulate the activity of redox-sensitive Rho GTPases in cell-based studies and facilitate guanine nucleotide dissociation *in vitro*, these GTPases are likely involved in a redox feedback loop.

Rac proteins and RhoA regulate NOS expression and activity. The activity and expression of the three NOS isoforms—eNOS, neuronal NOS (nNOS), and inducible NOS (iNOS)—are highly regulated (13). Whereas Rac proteins have been shown to regulate NOS expression and activity, RhoA regulates NOS expression (Figs. 5 and 6). Further, NOS bioavailability is, in part, attributed to its mRNA stability, which Rho GTPases can either enhance (Rac1) or reduce (RhoA) (45, 46). Rac1 promotes eNOS transcription through p21-activated kinase (PAK1), and RhoA reduces mRNA stability through Rho-associated protein kinase (ROCK) (37). Moreover, Rac1 and RhoA work in opposition to regulate NOS activity through the PI3K/Akt redox-sensitive pathway; RhoA inhibits this pathway, and Rac1 activates it (30, 37). Notably, Ras also modulates the PI3K/Akt pathway to regulate eNOS activity (35). As further evidence of RhoA/Rac1 crosstalk in NOS activation, Rac1 regulates cGMP-dependent kinase, which inhibits RhoA activation (37). The Rac1/PAK1 interaction further enhances eNOS activity by stimulating cell uptake of L-Arginine (37). Rac proteins may regulate NOS function in two additional ways, controlling localization and direct protein–protein interaction (31, 48). Rac2 and iNOS interact in a direct, GTP-independent manner, and Rac2 overexpression promotes iNOS localization to cytoskeletal complexes (31). Rac1 has been shown to directly interact with all of the NOS isoforms, and overexpression of $Rac1^{CA}$ enhances eNOS and nNOS activity, likely by direct interaction (31, 45, 48). As NOS isoforms interact with Rac isoforms with varying efficiencies depending upon cell type, stimulating

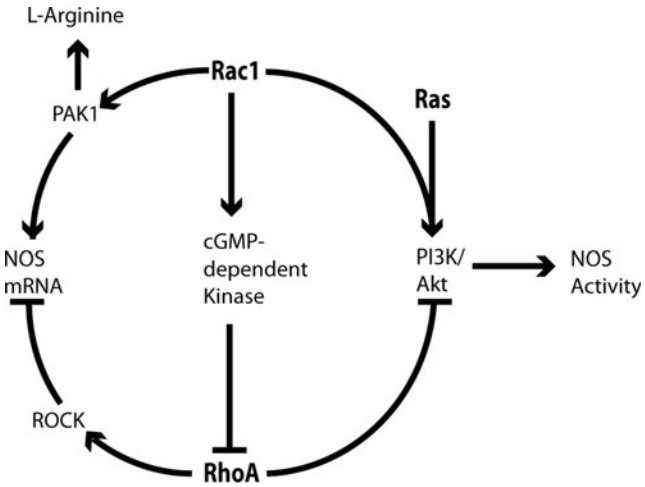

FIG. 5. GTPase crosstalk in NOS regulation. Shown below are the following: RhoA regulates NOS expression by decreasing mRNA stability through ROCK, and it decreases NOS activity through inactivation of the PI3K/Akt pathway (37, 45, 46). Rac1 enhances NOS mRNA expression through PAK1, and it increases NOS activity by direct interaction (45, 47, 48), activating the PI3K/Akt pathway (30), increasing cell uptake of L-Arginine, and downregulating RhoA activity through cGMP-dependent kinase (37). Ras also plays a role in NOS stimulation through the PI3K/Akt pathway (35). NOS, nitric oxide synthase; PAK1, p21-activated kinase; PI3K, phosphoinositide 3-kinase.

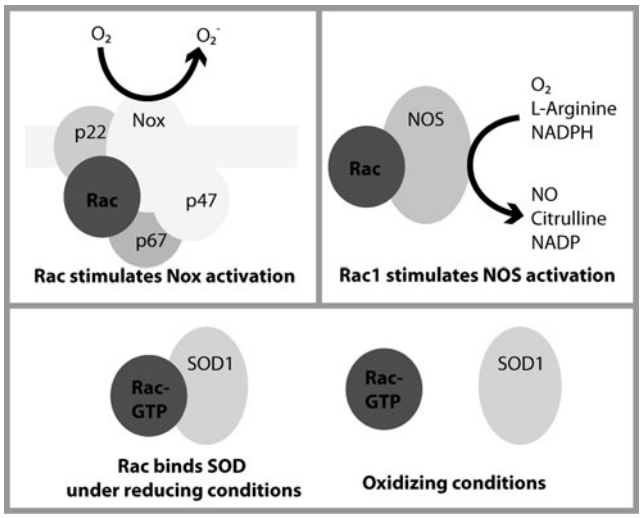

FIG. 6. Rac directly interacts with Nox, NOS, and SOD. Rac isoforms have been shown to directly enhance the activities of NOS and Nox isoforms (23, 45, 48). In several Nox isoforms, Rac must interact with the p67 subunit (and possibly flavocytochrome b558) for Nox to convert O_2 to $O_2^{\bullet-}$ (7, 23). In addition to evidence that Rac indirectly stimulates NOS activity, Rac1 has been shown to increase eNOS and nNOS activity through direct interaction, aiding in the conversion of O_2 to NO (45, 48). Finally, recent data show that Rac1 directly interacts with SOD1 in a nucleotide- and redox-dependent manner (16). eNOS, endothelial NOS; nNOS, neuronal NOS; $O_2^{\bullet-}$, superoxide; SOD, superoxide dismutase.

event, and Rac nucleotide status, this interaction may aid in proper compartmentalization for NO generation.

Rac proteins activate Nox. Rac proteins regulate Nox activity, as they can directly interact with and activate multiple Nox isoforms to produce $O_2^{\bullet-}$, including Nox1, 2, 3, and 4 (23). Rac1 is required for Nox1 activity in vascular smooth muscle cells, and Rac2 activates Nox2 in neutrophils (7, 11). Interestingly, Rap1A, an NKCD-motif-containing Ras subclass GTPase, has been implicated in Nox activation (11). Nox activity can be stimulated by multiple factors, including shear stress, angiotensin II, thrombin, insulin, and vascular endothelial growth factor (3, 6, 26). Various models have been proposed for Rac activation of Nox (Fig. 6), and data support Nox activation by GTP-bound Rac and GDP-bound Rac complexed to GDP dissociation inhibitor (GDI); the authors suggest that GDI association maintains the active Rac conformation even when GDP-bound, as Rac likely binds Nox in this conformation (7, 12). The common steps in the models for Rac1 activation of Nox are as follows: first, upon stimulation, Rac and the cytosolic Nox components translocate simultaneously, but independently, to the cell membrane (7, 11); next, likely through its switch I and insert motifs, Rac directly binds p67 to activate Nox (7). Controversial in these models is whether Rac1 also directly interacts with flavocytochrome b558, whether it activates Nox either through an adaptor function or mediates electron transfer during $O_2^{\bullet-}$ production, and whether the Rac insert region is required for Nox activation (7, 11). Depending on cell type, localization, and stimulating event, Rac-dependent Nox activation can promote various signaling pathways with physiological and pathological consequences, such as transcription activation, inflammatory responses, as well as cardiovascular and neurological diseases (46).

Rac1 directly interacts with SOD in a nucleotide- and redox-dependent manner. Recent evidence supports a role for Rac1 in regulating SOD1 (Fig. 6), a key antioxidant enzyme in the cell. In a detailed study, Harraz *et al.* showed that Rac1 interacts with SOD1 in a nucleotide-dependent manner, indicative of GTPase/effector interactions (Fig. 6) (16). As this study involved cell-based assays, our lab is using *in vitro* methods to quantitate this interaction and has validated its nucleotide dependence (unpublished observations). Harraz *et al.* showed that cell redox conditions also affect the Rac1/SOD1 interaction and suggested a feedback mechanism. However, the question remains whether the Rac1/SOD1 interaction enhances SOD1 activity, as in Nox and NOS. Interestingly, these experiments were performed in an amyotrophic lateral sclerosis (ALS) model, and SOD mutations that are common in ALS patients were proposed to uncouple the Rac1/SOD feedback mechanism and enhance cell toxicity. A redox regulatory role for the Rac1/SOD interaction in neural physiology and pathology is consistent with Rac regulation of NOS and Nox in key physiological and pathological pathways.

Conclusions

We have reviewed the cell-based literature demonstrating that RIs regulate Rho GTPase activity and certain Rho GTPases control the activities of the predominant redox-modulating enzymes in cells. As we have recently published a review on RNS regulation of Ras GTPases, we have only noted some of the most recent data. We speculate that Rac is a common control element for regulating cell redox state. Moreover, RhoA has been shown to play a key role in regulating NOS. We postulate that cell redox conditions (oxidative/reducing) regulate the activity of these GTPases; however, the role of these GTPases in controlling RI-regulating enzymes suggests a potential feedback mechanism.

Conclusions and Future Directions

Herein, we discussed the importance of RIs in modulating the activity of select Ras and Rho GTPases and the role of these GTPases in controlling the expression and activity of RI-regulating enzymes. Further, we have previously discussed RNS regulation of Ras in depth (10). As there is a discrepancy in the literature on whether Ras is activated by $1e^-$ (radical) and $2e^-$ (covalent) cysteine oxidation mechanisms, we propose that future experiments address this discrepancy. In addition, our data and a growing body of literature suggest that Rho GTPases are particularly susceptible to redox control given their unique redox-sensitive cysteine-containing p-loop motif. This redox-sensitive cysteine interacts with the bound nucleotide, and $1e^-$ and $2e^-$ redox modification at this cysteine enhances nucleotide exchange, likely by disrupting these interactions. Under physiological conditions, RIs likely activate Ras and Rho GTPases, whereas oxidative stress may promote their inactivation. To aid in discerning whether RI regulation of Rho GTPases is direct or indirect, we have identified redox-insensitive variants that better mimic the wild-type proteins. Moreover, cell-based studies should be

performed with endogenous RI sources, redox state characterization, and absent GTPase overexpression. Further, Rho and Ras subclasses also regulate expression and activity of key redox regulating enzymes. RhoA and Ras regulate NOS expression and activity, and Rap1A has been implicated in Nox regulation. Rac controls NOS and Nox, and Rac1 has recently been shown to directly interact with SOD1. Given that Rac1 and SOD1 are ubiquitously expressed proteins involved in numerous cell functions, future experiments should be directed to discerning the mechanism of and effects from this interaction. Further, as the literature is increasingly dedicated to redox-based signaling, perhaps future studies will better define how RIs regulate Ras and Rho GTPase activity and whether these GTPases are regulated by or control additional redox-modulating enzymes.

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Abbreviations Used

5-nitro-GDP = 5-nitro-guanine ribose diphosphate
ALS = amyotrophic lateral sclerosis
DTT = dithiothreitol
eNOS = endothelial NOS
GDI = GDP dissociation inhibitor
GSSG = oxidized glutathione
H_2O_2 = hydrogen peroxide
iNOS = inducible NOS
LMW-PTP = low-molecular-weight protein tyrosine phosphatase
MS = mass spectrometry
$NO^{\bullet}$ = nitric oxide
nNOS = neuronal NOS
NOS = nitric oxide synthase
Nox = NADPH oxidase
NIm-DP = 5-guanidino-4-nitroimidazole diphosphate
$O_2^{\bullet-}$ = superoxide
$^{\bullet}OH$ = hydroxyl radical
$ONOO^-$ = peroxynitrite
p-loop = phosphoryl-binding loop
PAK1 = p21-activated kinase
PI3K = phosphoinositide 3-kinase
RIs = reactive intermediates
RNS = reactive nitrogen species
ROCK = Rho-associated protein kinase
ROS = reactive oxygen species
SOD = superoxide dismutase