REVIEW

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Rho GTPases, oxidation, and cell redox control

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Abbreviations: BSO, L-buthionine sulfoximine; $CO_3^{\bullet-}$, carbonate radicals; DPI, diphenyleneiodonium chloride; DUB, deubiquitinating enzymes; eNOS, endothelial NOS; FeTTPS, 5,10,15,20-tetrakis(4-sulfonatophenyl) porphyrinato iron (III); GAPs, GTPase activating proteins; GDIs, guanine nucleotide dissociation inhibitors; GEFs, guanine nucleotide exchange factors; GSH, glutathione; GSH-Pxs, glutathione peroxidases; GSSG, oxidized glutathione; H_2O_2, peroxide; HAECs, human aortic endothelial cells; iNOS, inducible NOS; LMW-PTP, low-molecular weight protein tyrosine phosphatase; NAC, *N*-acetylcysteine; MDCK, Madin-Darby canine kidney cells; NFκB, nuclear factor-κB; NMuMG, normal mouse mammary gland epithelial cells; $NO^{\bullet}$, nitric oxide; $NO_2^{\bullet}$, nitrogen dioxide; nNOS, neuronal NOS; NOS, nitric oxide synthase; Nox, NADPH oxidase; NSCLC, non-small cell lung carcinoma cells; $O_2^{\bullet-}$, superoxide; $ONOO^-$, peroxynitrite; PAO; phenylarsine oxide; PTM, post-translational modification; RBD; Rho-binding domain; RNS, reactive nitrogen species; ROS, reactive oxygen species; SNO, s-nitrosothiols; SOD, superoxide dismutase

While numerous studies support regulation of Ras GTPases by reactive oxygen and nitrogen species, the Rho subfamily has received considerably less attention. Over the last few years, increasing evidence is emerging that supports the redox sensitivity of Rho GTPases. Moreover, as Rho GTPases regulate the cellular redox state by controlling enzymes that generate and convert reactive oxygen and nitrogen species, redox feedback loops likely exist. Here, we provide an overview of cellular oxidants, Rho GTPases, and their inter-dependence.

It has become increasingly clear that reactive oxygen and nitrogen species (ROS, RNS), once regarded simply as by-products of cellular metabolism, also function as regulators of numerous critical physiological processes, including cell survival,[1] proliferation,[2] differentiation,[3] migration, and adhesion.[4] To accomplish these activities, ROS and RNS modulate redox-sensitive proteins that control multiple kinase[5] and GTPase[6] signaling pathways. In particular, accumulating evidence suggests a role for ROS/RNS in the regulation of Ras and Rho family GTPases. Here, we highlight recent findings that demonstrate a role for ROS/RNS in the regulation of Rho GTPase activity.

Rho Family GTPases

Rho GTPases comprise 1 of 5 distinct classes of Ras superfamily GTPase proteins. The canonical members of the Rho subfamily are RhoA, Rac1, and Cdc42.[7] Rho GTPases function in a wide variety of cellular processes, including cell growth, motility, polarity, and adhesion.[8] Rho GTPases, like most Ras superfamily GTPases, cycle between the active GTP-bound and inactive GDP-bound states to modulate their binding to regulators and cellular targets (**Fig. 1**). The exchange of bound GDP for GTP (activation) is regulated by guanine nucleotide exchange factors (GEFs),[9] which catalyze GDP dissociation and promote GTP binding due to the higher cellular ratio of GTP to GDP. Once in their active GTP-bound state, Rho GTPases interact with a number of downstream effectors to promote their biological activities. Deactivation of the GTPases is catalyzed by GTPase activating proteins (GAPs), which stimulate hydrolysis of bound GTP to GDP and inorganic phosphate.[10] In addition, Rho GTPases are regulated by guanine nucleotide dissociation inhibitors (GDIs), which result in inactivation by sequestering the GDP-bound GTPase away from the membrane and stabilizing GDP binding.[11,12]

Rho GTPases can be modified by a variety of post-translational modifications (PTMs) that drive differences in localization and activity.[13] One such PTM is lipid modification. Rho proteins associate with cellular membranes upon isoprenoid lipid modification at their carboxyl-terminal CAAX (C, cysteine; A, aliphatic residue; X, variable residue) motifs.[14,15] RhoA, Rac1, and Cdc42 are all geranylgeranylated at the CAAX motif, which is required for their association with the inner leaflet of the plasma membrane and/or with internal membranes.[16] As membrane association is critical for downstream signaling,[17] dynamic PTMs and protein:protein interactions that affect membrane association can significantly affect Rho biological activity. For example, Rho GDIs recognize and sequester the geranylgeranyl lipid moiety,[18] preventing membrane association and inactivating the GTPase. Moreover, palmitoylation of Rac1 at Cys^{178} is critical for Rac1-mediated actin cytoskeleton remodeling.[19]

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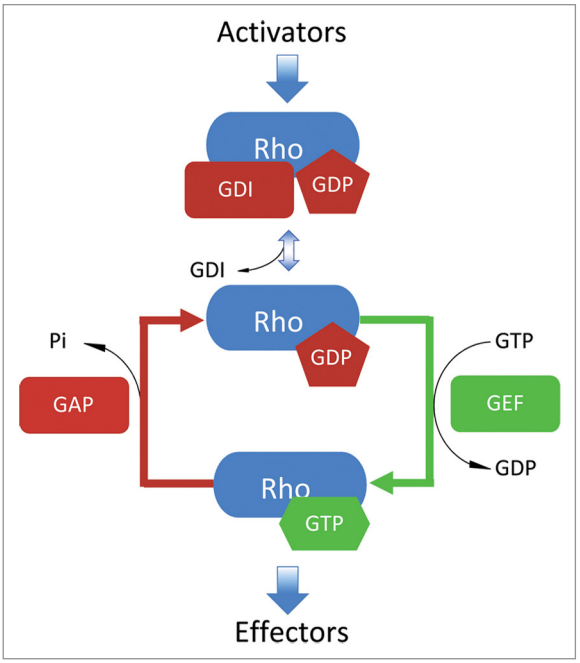


Figure 1. The GTPase cycle of Rho. Rho GTPases cycle between GTP-bound active states and GDP-bound inactive states. Guanine nucleotide dissociation inhibitors (RhoGDIs) extract lipid-modified Rho GTPases from cellular membranes to the cytosol and prevent GDP dissociation until released upon stimulus. Following signal input, the exchange of the bound GDP is facilitated by guanine nucleotide exchange factors (GEFs) that dramatically increase the dissociation rate of nucleotides. This promotes binding of GTP owing to its 10-fold higher in cellulo concentration vs. GDP. Once GTP-bound, active Rho is capable of binding downstream effectors and executing its biological functions. Rho remains active until GTP is converted to GDP due to its intrinsic hydrolytic capacity or through catalysis by GTPase activating proteins (GAPs).

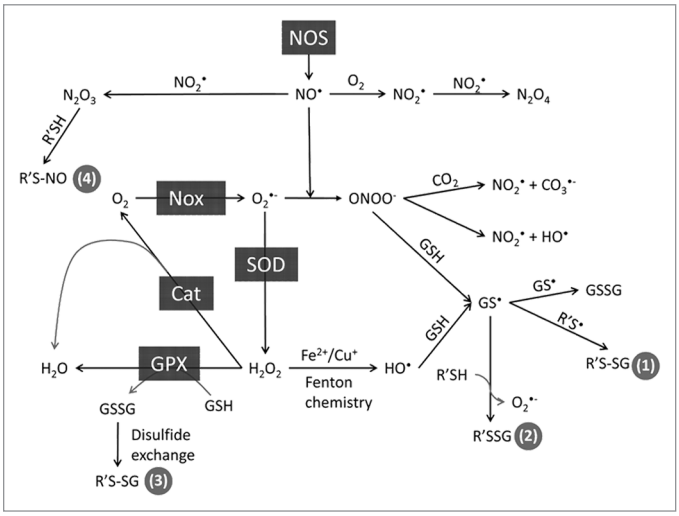


Figure 2. Generation and interconversion of ROS and RNS in cells. Select oxidation end products are presented, including radical-mediated glutathiolation (1), glutathiolation with $O_2^{\bullet-}$ production (2), disulfide exchange-mediated glutathiolation (3), and nitrosation by a non-radical pathway (4). Not all reactions shown are chemically balanced. Key: Cat, catalase; GPX, glutathione peroxidase; NOS, nitric oxide synthase; Nox, NADPH oxidase; SOD1, superoxide dismutase 1.

Mutation of Cys[178] to Ser, which prevents palmitoylation, results in decreased partitioning to the plasma membrane and reduced Rac1 activation. Rho GTPase membrane association is further regulated by interaction with other proteins and other nearby PTMs. Some Rho GTPases, such as RhoA, RhoG, and Cdc42, are phosphorylated at their C-terminal membrane-targeting domains near the prenylated CAAX motif,[20] which may be another mechanism of downregulation. While RhoA phosphorylation at Ser[188] increases its association with GDIs, which causes release from the membrane and inactivation, the biological effect of phosphorylation at this residue in RhoG and Cdc42 is unclear.[21,22]

Ubiquitination, including mono-, di-, and polyubiquitination, is another PTM that has been recently observed in Rho GTPases.[23] These modifications result in the addition of single or multiple ubiquitin moieties (76-residue protein) that affect protein localization, stability, and trafficking.[24] Ras was one of the first GTPases shown to be mono- and diubiquitinated. While ubiquitination of H-Ras promotes endosomal localization,[25,26] monoubiquitination at Lys[147] in K-Ras upregulates K-Ras activity by impairing GAP-mediated GTP hydrolysis.[27,28] Moreover, a subset of Rho GTPases is ubiquitinated. Of these, polyubiquitination of RhoA, Rac1, and Cdc42 result in their deactivation and degradation.[29-31] Rac1 is the only Rho family GTPase shown to be monoubiquitinated, and this modification affects Rac1 localization.[32] Ubiquitin is a highly regulated and reversible modification that can be removed by deubiquitinating enzymes (DUBs); however, Ras- and Rho-specific DUBs have yet to be discovered.

Cysteine oxidation represents yet another type of PTM in Ras superfamily GTPases. Although this form of redox regulation is often described as an "emerging" or "novel" mechanism of GTPase regulation, it was originally identified in Ras over 15 years ago.[33] What makes cysteine oxidation a unique PTM is that oxidation can occur absent direct enzymatic action. Rather, reactive and/or solvent exposed cysteines can be modified directly by reactive nitrogen (RNS) and oxygen (ROS) species (oxidants capable of cysteine oxidation are depicted in **Fig. 2**); however, whether enzymes can assist or catalyze thiol oxidation remains undetermined. While a variety of enzymes, including nitric oxide synthase (NOS), NADPH oxidase (NOX), and superoxide dismutase (SOD), produce oxidants that promote protein oxidation, the oxidants themselves have long been viewed as nonspecific hallmarks of oxidative stress. However, it has become increasingly apparent that specific proteins containing redox-sensitive cysteine residues are targeted under physiological conditions by both RNS and ROS, including nitrogen dioxide ($NO_2^{\bullet}$), nitric oxide ($NO^{\bullet}$), superoxide ($O_2^{\bullet-}$), peroxynitrite ($ONOO^-$), and peroxide (H_2O_2).[34-36]

Ras GTPases were among the first GTPases shown to be sensitive to RNS and ROS in cells.[33] Ras proteins contain a redox-sensitive NKCD motif. While covalent modification of this cysteine (Cys[118]) by oxidants does not alter Ras activity,[37,38] oxidants capable of generating a thiyl radical at Cys[118] (e.g.,

$NO_2^{\bullet}$) cause oxidation of the guanine nucleotide and enhance nucleotide exchange under physiological or mild-oxidizing conditions.[39,40] Given the >10-fold higher levels of cellular GTP compared with GDP, this enhanced exchange likely activates Ras.[41] In support of these findings, radical-mediated activation of Ras at Cys^{118} contributes to tumor maintenance in a pancreatic cancer cell line and in a severe combined immunodeficiency/beige mouse model.[42] However, a subset of Rho family GTPases has been observed to be sensitive to oxidants.[43,44] In Rho GTPases, the redox-sensitive motif has been determined to be located directly adjacent to the phosphoryl-binding loop (GXXXXGK[S/T]C) motif. As with Ras, Rho GTPases are sensitive to free radical-mediated oxidation and regulation; however, because the redox-sensitive thiols in Rho GTPases make direct contact with the bound nucleotide, 2-electron oxidation of the phosphoryl-binding loop thiols can also regulate Rho GTPase activity.

There is no known consensus sequence that can be used to predict which cysteines are redox-sensitive.[45] A cysteine residue is generally considered reactive to oxidants, or "redox-sensitive," if it has a depressed pK_a and is solvent accessible.[46,47] In general, a free cysteine has a pK_a of approximately 8.5, which populates the thiol form at physiological pH, and is less sensitive to oxidation than the thiolate (S^-) form.[48] For example, while peroxynitrite can oxidize the cysteine thiol, it is more reactive with the thiolate. The radical-mediated breakdown products of peroxynitrite are even more reactive and likely to oxidize cellular thiols.[49] Moreover, nitric oxide and peroxide are believed to react primarily with the thiolate form of cysteines as they react too slowly with thiols to be physiologically relevant.[50] However, an altered pK_a is not always a prerequisite for redox sensitivity, especially for radical-mediated reactions, as Ras Cys^{118} does not have an altered pK_a despite its regulation by free radical-mediated oxidants.[51] Furthermore, as oxidants are generally short-lived and confined to the compartment in which they are generated, localization of the enzymes that generate oxidants with their target proteins is often critical for regulation by ROS and RNS.

In this review, we present fundamental concepts associated with redox signaling, with a specific emphasis on cysteine oxidation, and describe the current state of the field regarding the redox regulation of Rho GTPases.

Generation of ROS and RNS in Cells and Antioxidant Defense

Varying levels of oxidative stress can occur in cells when the cellular redox potential becomes oxidizing. Dysregulation of the cellular redox potential is correlated with elevated levels of ROS and RNS, which contributes to and promotes a variety of diseases, including cancer,[52] neurodegeneration,[53] atherosclerosis,[54] diabetes,[55] and aging.[56,57] As such, ROS and RNS are often considered damaging to cellular components, including proteins, lipids, and DNA. To provide appropriate background for the reader on the currently understood role of ROS and RNS in the regulation of Rho GTPases, we will first give a brief overview of cellular ROS/RNS production and the cellular antioxidant defense system.

Nitric oxide is perhaps the best-known cellular RNS and is produced by a class of enzymes called nitric oxide synthases (NOSs). There are 3 NOS isoforms: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS), all of which function as homodimers to generate nitric oxide ($NO^{\bullet}$) from L-arginine.[58] Some functions of nitric oxide produced by NOS include the regulation of blood pressure by controlling vasodilation, inflammation, and erectile function.[58] Endothelial NOS is constitutively expressed and localized to cell membranes.[59] When activated, eNOS can produce low levels of nitric oxide. Neuronal NOS is a cytosolic protein that is found primarily in the central nervous system.[60] Activation of eNOS and nNOS is regulated by calcium-bound calmodulin,[61] interactions with various effector proteins,[62] and phosphorylation.[63] Lastly, iNOS expression is induced in macrophages and other cell types; however, unlike eNOS or nNOS, iNOS is constitutively active once expressed.[64] The functional role of iNOS is to produce nitric oxide to regulate the immune response, including inhibition of iron-sulfur proteins, oxidation of invading pathogen DNA, and oxidative damage of tumor cells.

A primary source of cellular ROS exists within the mitochondria. All aerobic organisms require oxygen for oxidative phosphorylation, and the mitochondria are central organelles that generate a number of cellular oxidants.[65] Moreover, nitric oxide regulates oxygen consumption, metabolism, and ROS production in the mitochondria[66] and can be generated through NOS-dependent and -independent pathways.[67] One of the most common ROS generated by the mitochondria is superoxide ($O_2^{\bullet-}$), which is produced due to the incomplete reduction of molecular oxygen (O_2). Superoxide is a charged molecule and does not readily diffuse across membranes,[68,69] which limits where it can react with cellular biomolecules. Furthermore, in aqueous solution, superoxide undergoes a fast dismutation reaction (rate constant of $5 \times 10^5\ M^{-1}s^{-1}$ at pH 7.0) to peroxide (H_2O_2).[70] Superoxide dismutase, an enzyme capable of reducing superoxide to peroxide, speeds up this reaction nearly 10^4-fold.[71] In the mitochondria, SOD2 is maintained at a high concentration.[72,73] However, the product of this reaction, H_2O_2, can easily cross the membrane and move into the cytoplasm.[74]

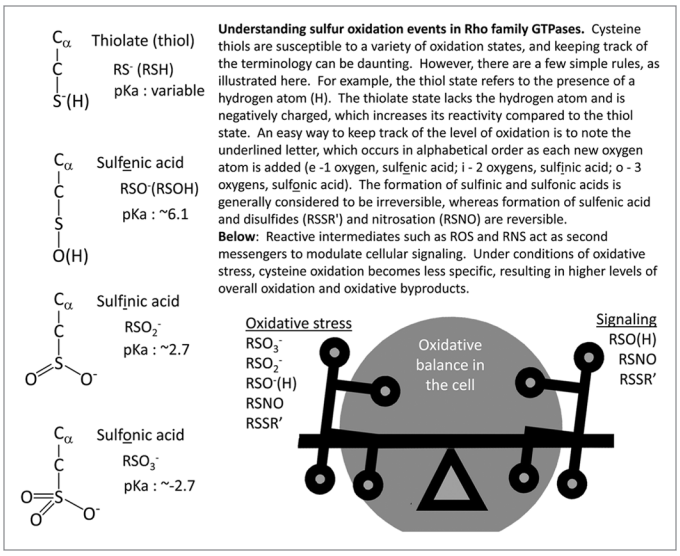
Phagocytes are a common source of cellular ROS and can produce superoxide in "bursts" due to the action of NOXs.[75] There are currently 7 known NOX enzymes (Nox1/2/3/4/5, Duox1, and Duox2). NOX1, NOX2, and NOX3 all require the Rac1 GTPase for activation.[76] NOX isoforms are highly regulated and tissue-specific. In phagocytes, cytochrome b559 (a heterodimer consisting of NOX2 ([formerly $gp91^{phox}$] and $p22^{phox}$) combines with $p47^{phox}$, $p67^{phox}$, and Rac1 to form the membrane-associated NOX complex, which catalyzes the 1-electron reduction of molecular oxygen to superoxide. A major role of the NOX complex is to eliminate invading pathogens by oxidation.[77] In addition, superoxide generated in the arteries by NOX2 and NOX4 compete with the antihypertensive effects of nitric oxide by reacting with nitric oxide to form peroxynitrite.[78]

The cell has also evolved a network of antioxidant enzymes and small molecules to protect the cell from oxidative damage. SOD is an abundant antioxidant enzyme.[79] Three distinct isoforms of SOD exist: SOD1, a dimeric protein that binds copper and zinc and is localized in the cytosol;[80,81] SOD2, a homotetrameric protein that binds 1 manganese ion per subunit and is localized in the mitochondria;[82] and SOD3, a homotetrameric protein that binds copper and zinc and is anchored to the extracellular matrix.[83,84] SODs efficiently reduce superoxide to peroxide.[80] Peroxide, in turn, can be reduced to water by catalases and glutathione peroxidases (GSH-Pxs) (**Fig. 2**).[85] In addition, GSH-Pxs can reduce lipid peroxides to their respective lipid alcohols.[86,87] Other antioxidant enzymes include thioredoxin reductases, peroxiredoxins, and glutaredoxins, all of which function in the removal of peroxide.[88]

Common cellular small molecule antioxidants include glutathione, ascorbic acid, tocopherol, and β-carotene. Glutathione is a tripeptide that contains a cysteine thiol group and is the major cellular antioxidant. The cellular concentration of glutathione in cells ranges between 0.5 and 10 mM, whereas oxidized glutathione is reported to range from 5–50 μM.[89] Proper regulation of the ratio of reduced to oxidized glutathione (GSH:GSSG) is critical for managing the cellular redox potential.[90] GSH functions with GSH-Pxs to reduce peroxides in the cell and can reduce other oxidized species. Ascorbic acid, more commonly known as Vitamin C, scavenges free radicals in the cytosol, whereas tocopherol (vitamin E) protects cellular membranes from oxidative damage.[91] β-carotene serves to scavenge radicals as well, including peroxyl, hydroxyl, and superoxide radicals.[92]

Reactive Oxygen and Nitrogen Species as Second Messengers

While ROS and RNS are generally grouped into a single category of reactive intermediates, it is important to understand that there are 2 classes of oxidants, 2-electron and 1-electron oxidants. A predominant cellular 2-electron oxidant is hydrogen peroxide.[47] One-electron oxidants, such as nitrogen dioxide, generate thiyl radical intermediates. In general, free radical oxidants have a high redox potential and react faster with cellular thiols than 2-electron (non-radical) oxidants. Oxidation can result in a variety of cysteine oxidative byproducts, thereby altering the cellular redox state or protein function (**Fig. 2**).[93,94] Importantly, ROS and RNS can interact with cell signaling proteins (phosphatases, kinases, etc.) and function as second messengers downstream of a variety of signaling stimuli.[35] However, it is important to note that oxidation observed in vitro does not always correlate with oxidation in vivo, as in vitro experiments tend to have few substrates in solution, which leaves the oxidant little competition. Moreover, reaction of protein thiols with cellular oxidants is dependent on thiol accessibility, reactivity, spatial and temporal localization to oxidants, and enzymes that catalyze thiol oxidation and reduction.[95]



Understanding sulfur oxidation events in Rho family GTPases. Cysteine thiols are susceptible to a variety of oxidation states, and keeping track of the terminology can be daunting. However, there are a few simple rules, as illustrated here. For example, the thiol state refers to the presence of a hydrogen atom (H). The thiolate state lacks the hydrogen atom and is negatively charged, which increases its reactivity compared to the thiol state. An easy way to keep track of the level of oxidation is to note the underlined letter, which occurs in alphabetical order as each new oxygen atom is added (e -1 oxygen, sulfenic acid; i - 2 oxygens, sulfinic acid; o - 3 oxygens, sulfonic acid). The formation of sulfinic and sulfonic acids is generally considered to be irreversible, whereas formation of sulfenic acid and disulfides (RSSR') and nitrosation (RSNO) are reversible.
Below: Reactive intermediates such as ROS and RNS act as second messengers to modulate cellular signaling. Under conditions of oxidative stress, cysteine oxidation becomes less specific, resulting in higher levels of overall oxidation and oxidative byproducts.

Some examples of common ROS are peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$), and hydroxyl radical ($HO^{\bullet}$). Examples of common RNS are nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$), peroxynitrite ($ONOO^-$), and dinitrogen trioxide (N_2O_3; **Fig. 2**; see Glossary). For these molecules to function as second messengers in cellular signaling, they must be generated in response to a specific stimulus, be short-lived, specifically target effectors, and transiently and reversibly affect signaling. Superoxide and peroxide are considered to function as second messengers but are less reactive than some other ROS, such as the hydroxyl radical. The hydroxyl radical lacks specificity as it reacts with virtually all biomolecules at the rate of diffusion. Aside from peroxynitrite and dinitrogen trioxide (N_2O_3), other cellular RNS that oxidize thiols are free radicals. Nitrogen dioxide, rather than nitric oxide, is thought to be the major radical-mediated cellular RNS. The thiyl radical, once formed, can react with nitric oxide to generate nitrosothiols (SNO). Peroxynitrite undergoes homolytic cleavage to generate nitrogen dioxide and carbonate radicals ($CO_3^{\bullet -}$) in the presence of CO_2, which can directly oxidize thiols; however, dinitrogen trioxide and the nitrosonium ion (NO^+) are capable of nitrosating thiols through slower 2-electron mechanisms[96] to generate nitrosothiols (RSNO). For a detailed review of the interconversion of ROS and RNS, we direct readers to a previous review.[97]

Protein thiols in cysteine residues are among the most common sites of protein oxidation. Reversible oxidation states of protein thiols in vivo include oxidation to sulfenic acid (RSO^-), nitrosation (RSNO), glutathiolation (RSSG; mixed disulfide bonds), and disulfide bonds (RSSR'; intramolecular disulfide bonds).[98] Sulfinic acid (RSO_2^-), which has been regarded as an irreversible modification, has been shown to be reduced by an enzyme class termed sulfiredoxins; however, sulfiredoxins have only been shown to reduce sulfinic acids in specific peroxiredoxins.[99] Sulfonic acid (RSO_3^-) is another consequence of thiol oxidation; however, this oxidation state is irreversible and often results in proteasome-mediated degradation.[100]

Redox Regulation by Rac

Small GTPases of the Rho family both regulate and are regulated by ROS and RNS. It has been well established that Rac GTPases modulate the cellular redox state through regulating the assembly and activation of the NOX complex.[101] In particular, Rac1 and Rac2 are involved in the activation of NOX1, NOX2, and NOX3.[101-103] This function of Rac, first demonstrated in neutrophils and other phagocytic cells and later expanded to non-phagocytic cells,[104] is important in both physiological[105] and pathophysiological[106-108] settings.

Rac1 has been shown to associate with many enzymes necessary for maintaining redox homeostasis in cells. SOD1, but not SOD2, directly associates with Rac1 and has been proposed to regulate Rac1/NOX2 activation in a redox-dependent fashion.[109] Rac1 and SOD1 can be immunoprecipitated from whole organ lysates of mice, including the brain, liver, kidney, and heart. In addition, in vitro-purified Rac1 can directly associate with SOD1.[109] Intriguingly, the interaction between Rac1 and SOD1 appears dependent on both the bound guanine nucleotide and oxidation state of Rac1. In this study, Rac1 was purified from bacteria and used to pull native SOD1 from brain tissue lysates. In the absence of reducing agents, Rac1-GDP co-immunoprecipitated with SOD1, whereas in the presence of reducing agent (300 μM DTT), only Rac1-GTP co-immunoprecipitated with SOD1. While this result has interesting implications regarding redox-dependent interactions between Rac1 and SOD1, the oxidation state and sites of Rac1 oxidation were not determined. Thus, this study raised new questions but did not answer questions regarding the regulation or consequences of oxidation on Rac1/SOD1 binding.

Further interplay of Rac1 and ROS/RNS production involves the regulation of NOS activity. Selvakumar et al. observed a concomitant increase or decrease in nitric oxide and superoxide production in primary human aortic endothelial cells (HAECs) when Rac1 was activated or inhibited, respectively.[110] Consistent with these observations, Rac1 can interact with and regulate the activity of the eNOS and nNOS. Rac2, however, interacts with iNOS, which is required for nitric oxide generation following phagocytosis.[111]

A biologically relevant downstream effect of ROS is activation of nuclear factor-κB (NFκB), a transcription factor that controls the expression of a wide spectrum of genes, especially genes involved in inflammation and the immune response.[112,113] Many Rac1-induced tumorigenic properties have been ascribed to Rac1-mediated ROS production and NFκB activation.[113-115] Although the majority of the experimental evidence argues for an activating role of ROS, mostly H_2O_2, on NFκB, inhibition of basal NFκB activity by cadmium, a heavy metal that leads to rapid and transient ROS generation, has also been reported.[113] Interestingly, depending on the cellular context, NFκB reciprocally regulates ROS in a bidirectional manner (**Fig. 3**). During inflammation, activated NFκB facilitates the production of ROS through the induction of NOX2 to strengthen the immune defense.[116] However, when cellular ROS accumulation becomes toxic, NFκB can prevent oxidative damage through the transcriptional activation of SOD2.[117,118]

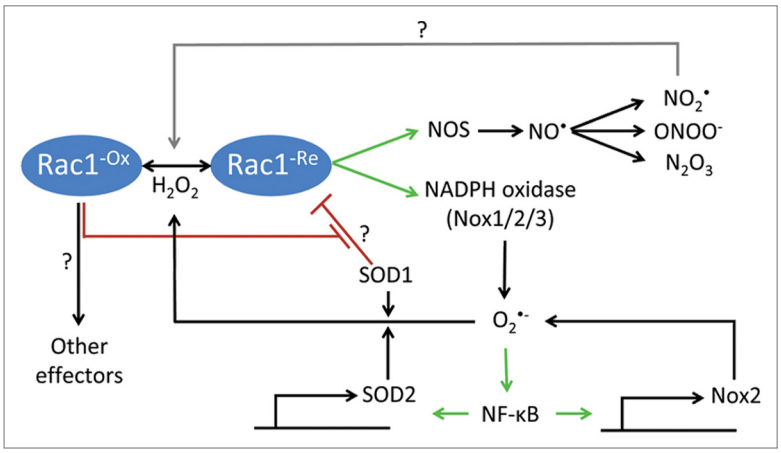


Figure 3. Rac1-mediated oxidant production. Rac1 assembles and activates NADPH oxidases 1/2/3 (Nox1/2/3), which produce superoxide ($O_2^{\cdot-}$). $O_2^{\cdot-}$ activates nuclear factor-κB (NFκB), a transcription factor that facilitates the transcription of, among other genes, redox-related enzymes, such as Nox2 and superoxide dismutase 2 (SOD2). Nox2 can further enhance the production of oxidants, while SOD2 converts highly reactive $O_2^{\cdot-}$ to less reactive hydrogen peroxide (H_2O_2), the latter activating Rac1 via yet uncharacterized mechanisms. Under reducing conditions, SOD1 binds Rac1 and has been proposed to inhibit its GTPase activity. Oxidation of Rac1 by H_2O_2 uncouples SOD1-Rac1 interaction. Whether and how oxidized Rac1 further signals to other effectors is still not fully understood. In a similar vein to superoxide production, Rac1 interacts with and activates nitrogen oxide synthases (NOS), promoting the synthesis of nitric oxide ($NO^{\cdot}$). $NO^{\cdot}$ can be converted into various reactive nitrogen species (RNS), including nitrogen dioxide ($NO_2^{\cdot}$), peroxynitrite ($ONOO^-$), and dinitrogen trioxide (N_2O_3), which possibly regulate Rac1 activity.

Redox Regulation of Rac

In contrast to the vast amount of literature documenting Rac-mediated ROS production,[101-108] relatively few studies have investigated the role of ROS/RNS in regulating Rac activity. Nagase et al. have demonstrated ROS-mediated activation of Rac1 in rat cardiomyocytes. They found that L-buthionine sulfoximine (BSO), which depletes intracellular glutathione, increased the active form of Rac1, and this effect was blocked by the antioxidant *N*-acetylcysteine (NAC).[119] Consistent with their findings with BSO, H_2O_2 also activated Rac1.[119] Similarly, H_2O_2 treatment led to a robust increase in GTP-bound Rac1 in neutrophils derived from C57/BL6 control mice.[120] Chronic exposure of normal mouse mammary gland epithelial (NMuMG) cells to H_2O_2 not only elevated Rac1 activity but also greatly enhanced cell invasiveness.[121] These lines of evidence suggest that a positive feedback loop exists between Rac GTPases and ROS, which reinforces their effects on cells (**Fig. 3**). However, these studies have not tested whether the effects of ROS on Rac1 are direct or indirect. In addition, the effects of endogenous ROS on Rac activity have yet to be tested.

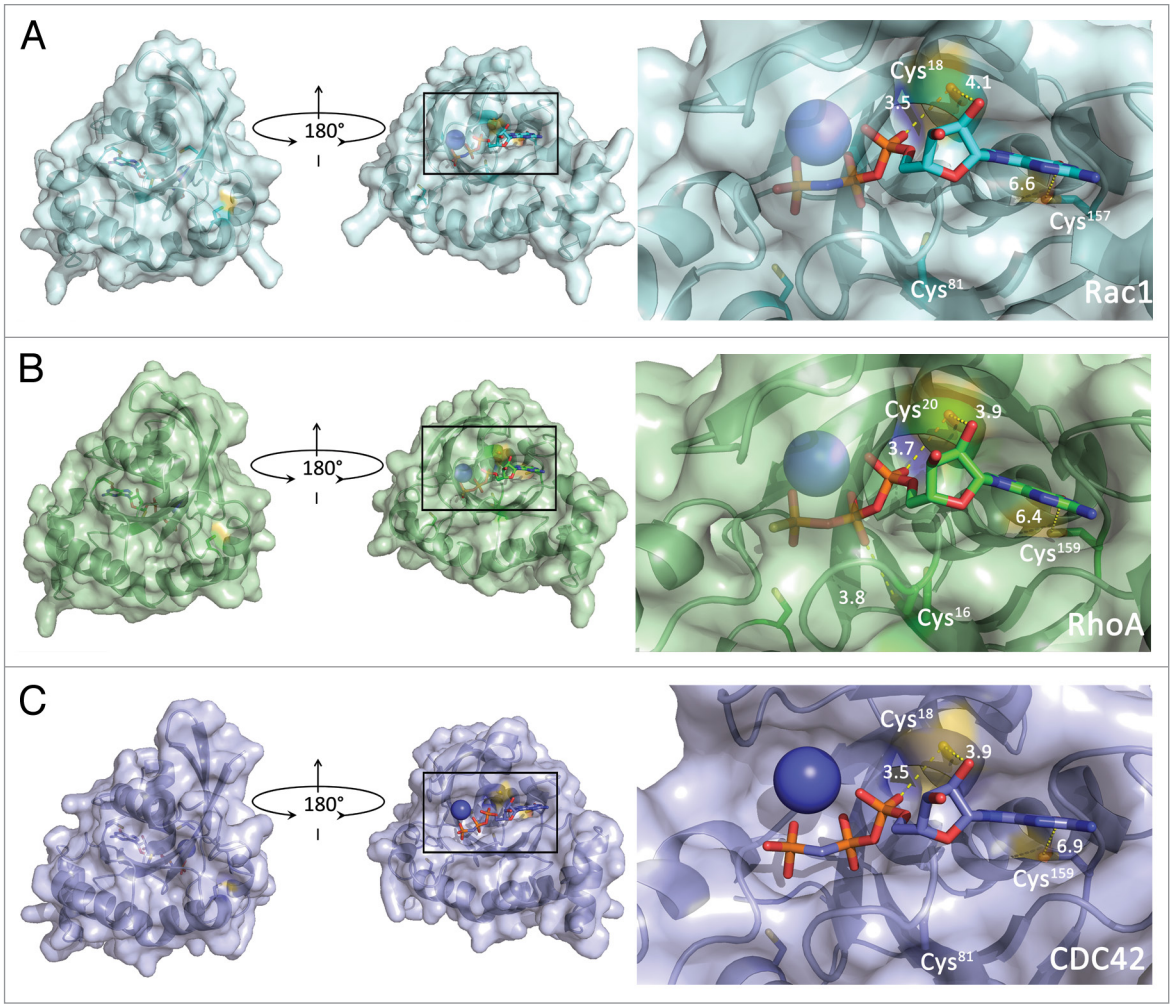


Figure 4. Surface rendering depicting cysteine solvent-accessible residues (yellow) in Rho family GTPases (see **Table 1**). (**A**) Left panel, Rac1 (PDB 3TH5) flipped by 180° around the y-axis. Right panel, close-up of the nucleotide-binding site with the redox-sensitive thiol (Cys^{18}) shown. Other local thiols are labeled (note that Cys^{81} is not solvent-accessible). Distances are presented in Å from the reactive thiol to the nucleotide. (**B**) RhoA surface rendering (PDB 1A2B). Images are as in (**A**). The close-up of the nucleotide-binding pocket (right panel) shows the redox-sensitive thiol (Cys^{20}) as well as other local thiols. (**C**) Cdc42 surface rendering (PDB 2QRZ). Images are as in (**A**). The close-up of the nucleotide-binding pocket (right panel) highlights $Cys.^{18}$

Many Rho family GTPases contain a distinct cysteine-containing motif (GXXXXGK[S/T]C) located adjacent to the phosphoryl-binding loop (p-loop). In Rho GTPases containing this motif, the redox-sensitive thiol (Cys^{18} in Rac1 and Cdc42, Cys^{20} in RhoA) makes direct contact with the bound nucleotide;[122] therefore, any modification of this cysteine has been predicted to affect the nucleotide binding properties of these GTPases (see **Fig.** 4).

Rho family GTPases containing this redox-sensitive motif include RhoA, RhoB, RhoC, RhoG, all Rac isoforms, and Cdc42 (for sequence comparison of the redox-sensitive motifs in Ras and Rho family GTPases, see ref. 6). The seminal papers that first identified the redox-sensitive properties of this motif in the canonical Rho family members (RhoA, Rac1, and Cdc42) showed that exposure to free radical oxidants (nitrogen dioxide and superoxide) resulted in increased nucleotide dissociation, similar to that observed for Ras.[43,44] In these studies, the in vitro-purified GTPases preloaded with [^{3}H]GDP were exposed to various ROS and RNS and monitored for their ability to retain nucleotide binding in the presence of unlabeled GDP. Whereas the non-radical oxidant peroxide stimulated guanine nucleotide dissociation in RhoA, Rac1, and Cdc42 by 10-fold under the conditions tested, the rate of guanine nucleotide dissociation was dramatically enhanced by 500- to 600-fold upon exposure to superoxide, nitrogen dioxide, and hydroxyl radicals. While these studies demonstrated that exposure of Rac1, RhoA, and Cdc42 to ROS and RNS can enhance nucleotide exchange and upregulate their function similar to the action of GEFs, oxidative modification of the GTPases was not evaluated.

We characterized the pK_a of the redox-sensitive thiol in Rac1 and determined whether cysteine oxidation alters Rac1 activity in vitro. We found that Cys^{18} in Rac1 has a depressed pK_a that populates the more reactive thiol anion (thiolate) at physiological pH, rendering Rac1 susceptible to thiol oxidation

Table 1. Solvent accessibility of cysteine sulfur atoms in Rac1, RhoA, and Cdc42

Thiol accessibility (Rac1/Cdc42, RhoA)	$Rac1^{GTP}$ PDB 3TH5	$Rac1^{GDP}$ (Zn-bound) PDB 2P2L	$RhoA^{GTP}$ PDB 1A2B	$RhoA^{GDP}$ PDB 1FTN	$Cdc42^{GTP}$ PDB 2QRZ	$Cdc42^{GDP}$ PDB 1AN0
Cys^{6}, NA	0.0	0.0	NA	NA	0.0	0.0
NA, Cys^{16}	NA	NA	0.0	0.0	NA	NA
Cys^{18}, Cys^{20}	8.7	0.0	4.1	0.0	0.0	0.0
Cys^{81}, Cys^{83}	0.0	0.0	0.0	0.0	0.0	0.0
Cys^{105}, Cys^{107}	9.8	0.56	3.9	1.9	0.0	0.05
Cys^{157}, Cys^{159}	0.70	0.45	1.2	0.04	0.21	0.13
Cys^{178}, NA	ND	35	NA	NA	6.0	3.7

Solvent accessibility (in square angstroms) of cysteine thiol atoms determined using nAccess.[107] Numbering in the left column is for Rac1 and Cdc42, with numbering for RhoA listed after the comma. nAccess was executed in the presence of heteroatoms, including the bound nucleotide. Only Cys^{18} (Cys^{20}) shows differential protection in the presence of nucleotide, displays nucleotide dependence, and increased accessibility in the absence of nucleotide. For comparison, the solvent accessibility of the sulfur atom in an Ala-Cys-Ala tripeptide is ~60 $Å^2$. ND, No data; NA, Not available.

(Hobbs et al.; in submission). Consistent with these findings, Cys^{18} was selectively oxidized by glutathione in vitro at a mildly acidic pH of 6.5. As physiological pH ranges from 6.7–7.4,[123] we used a slightly acidic pH to promote reaction with Rac1 thiol(s) that possess a depressed pK_a. Rac1 glutathiolation at Cys^{18} caused a significant (~230-fold) enhancement in the rate of intrinsic guanine nucleotide dissociation. Further, we generated a $Rac1^{C18D}$ variant to mimic sulfinic acid (RSO_2^-) oxidation at this site and observed greatly enhanced nucleotide exchange (220-fold), similar to that observed for glutathiolated Rac1, indicating that oxidation of Cys^{18} can significantly accelerate nucleotide exchange. However, as exposure of Rac1 to peroxide can increase intrinsic nucleotide dissociation by 10-fold,[44] we hypothesize that the effects of Rac1 oxidation are dependent on the oxidation state. The bulkier charged states, such as glutathiolation, result in greatly increased rates of nucleotide exchange and higher levels of activation relative to peroxide-mediated oxidation. Consistent with these results, Rac^{C18D} is highly activated in Swiss-3T3 cells and promotes lamellipodia formation (Hobbs et al.; in submission).

As cysteine oxidation of proteins is dependent on thiol accessibility, we used the program nAccess[124] to determine the solvent accessibility of cysteine residues in RhoA, Rac1, and Cdc42. **Table 1** lists the surface accessibility of all thiols in RhoA, Rac1, and Cdc42. While we find that thiol accessibility is dependent on the nucleotide bound state and interaction with regulators and effectors, we predict that Cys,[18] Cys,[105] and Cys^{178} (Rac1 numbering) are the most likely cysteine residues in Rac1 susceptible to oxidation based on solvent accessibility criterion alone. However, our data indicate that neither Cys^{105} nor Cys^{178} have a significantly altered pK_a. Moreover, Cys^{105} does not make direct interactions with the nucleotide or regions critical for effector and/or regulator recognition; therefore, if this cysteine becomes oxidized, Rac1 activity is unlikely to be altered. As Cys^{178} is modified by palmitic acid in vivo, it is possible that this cysteine will be protected from ROS/RNS modification, or conversely, that oxidation may prevent Rac1 palmitoylation. This, in turn, may modulate membrane association and/or kinetics. While oxidation of the C-terminal cysteines in Ras has been shown to regulate palmitate turnover,[125] the effect of oxidants on Rac1 lipidation has not been studied.

In contrast to our observations on Rac1, it has recently been observed that treatment of in vitro-purified Rac2 with a 10^5-fold excess of oxidized glutathione at a pH of 8.0 causes oxidation of Cys^{157}.[126] Using site-directed mutagenesis to generate individual Cys > Ala variants, the authors suggested that Rac2 was specifically glutathiolated at Cys^{157} in vitro. However, these experimental conditions likely result in nonselective oxidation of cysteines in Rac1. Given that Cys^{157} lies at the base of the nucleotide binding pocket and is inaccessible to the solvent (**Table 1**), it is unlikely that glutathiolation of Cys^{157} would be observed over Cys^{18} unless the protein fold was perturbed.

Redox Regulation by RhoA

Whereas Rac1 can directly stimulate ROS production via NOX activation and RNS production via NOS activation, RhoA can regulate RNS production via NOS expression levels. For example, activated RhoA negatively regulates eNOS expression in human endothelial cells,[127] bovine aortic endothelial cells,[128] and murine endothelial cells.[129] RhoA has also been shown to suppress iNOS expression and cellular nitric oxide levels in rat vascular smooth muscle cells,[130] in the normal human liver cell line AKN-1, and in the human non-small cell lung carcinoma (NSCLC) cell line A-549.[131] Therefore, RhoA appears to be actively engaged in downregulating NOS expression, which will reduce nitric oxide levels. As will be discussed below, addition of exogenous peroxynitrite to bovine aortic endothelial cells can promote RhoA activation.[128] If this is true in general terms, RhoA may represent a node of negative feedback on RNS production. However, whether RhoA is involved in ROS regulation is still unknown. Thus, whereas Rac1 promotes oxidant formation, Rho can downregulate a subset of oxidant-generating enzymes. Rac and Rho have long been known to have antagonistic roles and perhaps this is yet another example.

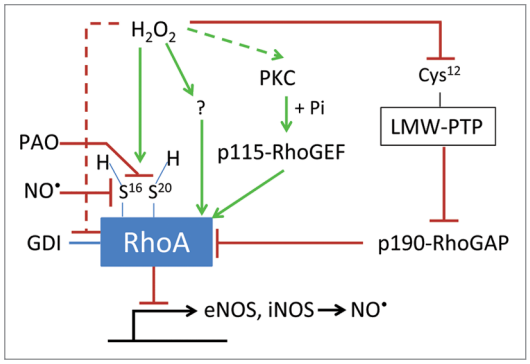


Figure 5. Redox-dependent regulation of RhoA. RhoA can be either activated or inactivated by oxidants. Reported modes of activation include: (1) PKC-dependent phosphorylation and activation of p115-RhoGEF; (2) release of GDI from RhoA; and (3) direct oxidation followed by reduction of cysteines 16 and 20. Reported modes of deactivation include: (1) phosphorylation and inactivation of p190-RhoGAP due to decreased activity of low-molecular weight protein tyrosine phosphatase (LMW-PTP) upon oxidation of an active site cysteine; (2) thiol cross-linking of cysteines 16 and 20 by phenylarsine oxide (PAO); and (3) NO•-induced RhoA S-nitrosation. However, it is still unclear whether oxidation of RhoA alters its interaction with downstream effectors.

Redox Regulation of RhoA

The pioneering work of Nimnual et al. established a model whereby the cellular redox state can be coupled to actin cytoskeleton dynamics through Rho GTPases.[132] In HeLa cells, the ability of constitutively active Rac1^{G12V} to induce membrane ruffling and integrin-stimulated cell spreading was attributed to Rac1-mediated redox-dependent oxidation in addition to the inactivation of low-molecular weight protein tyrosine phosphatase (LMW-PTP), which leads to the phosphorylation and activation of p190-RhoGAP.[132] The subsequent downregulation of RhoA activity thereby allowed Rac1 activity to proceed unimpeded (**Fig. 5**). These results required the presence of the "Rho insert" region (residues 124–135), which is necessary for Rac interaction with NOX. In contrast, addition of the antioxidant NAC, which replenishes glutathione stores, prevented Rac-mediated downregulation of RhoA-GTP, as did the NOS inhibitor diphenyleneiodonium chloride (DPI). DPI treatment prevented oxidation and inactivation of LMW-PTP as well as the phosphorylation and activation of p190-RhoGAP. In addition, DPI converted Rac1-mediated lamellipodia formation to the formation of dorsal ruffles and failed to downregulate RhoA-GTP levels. The dorsal ruffle phenotype is consistent with impairment in Rac-mediated ROS production. Finally, hydrogen peroxide was sufficient to decrease RhoA-GTP levels in a manner dependent on the insert domain of Rac, suggesting that Rac-mediated ROS generation was required. In contrast, ectopic expression of the hydrogen peroxide-scavenging enzyme catalase inhibited integrin-stimulated cell spreading. This inhibitory effect of catalase was rescued by non-oxidizable LMW-PTP^{C12S}. Collectively, these results strongly support the hypothesis that Rac-mediated organization of the actin cytoskeleton is regulated, in part, through Rac-induced ROS. This decreases RhoA-GTP levels due to the redox-mediated activation of p190-RhoGAP, a key RhoGAP. Similar results were later obtained in K-Ras-transformed normal rat kidney cells where DPI treatment was able to restore both RhoA-GTP levels and actin stress fiber formation that had been downregulated due to K-Ras activation of Rac1/NOX1.[133]

Another RhoA deactivation mechanism was revealed by Zuckerbraun et al.,[134] who showed that exogenously added nitric oxide reduced RhoA activity, as determined by decreased GTP binding in GST-Rhotekin-RBD pulldowns and by serum-stimulated stress fiber formation in rat aortic vascular smooth muscle cells. Direct modification of RhoA by S-nitrosation was verified using an anti-nitrosocysteine antibody. S-nitrosation was found to impair GTP binding, which consequently hinders RhoA activation. However, the exact site of S-nitrosation was not determined.

Finally, phenylarsine oxide (PAO) has been shown to deactivate RhoA. PAO can crosslink vicinal thiol groups and is a known phosphatase inhibitor.[135] Treatment of human colonic Caco-2 cells with PAO exhibited a dose-dependent reduction in actin stress fibers, a phenotype consistent with reduced RhoA activity. Moreover, pull-down experiments revealed a PAO concentration-dependent decrease in the levels of GTP-bound RhoA. The authors performed nucleotide dissociation and association assays in vitro and observed a loss of nucleotide binding in RhoA but not Rac1 upon exposure to PAO.[135] Mass spectrometry revealed that PAO crosslinked 2 vicinal cysteines in RhoA to occlude nucleotide binding. These effects were specific to RhoA but not Rac1 as only Rho GTPases (RhoA, RhoB, RhoC, and RhoE) possess vicinal cysteines (Cys^{16} and Cys^{20}) within the guanine nucleotide-binding region. In contrast, Rac1 contains only a single cysteine (Cys^{18}) and reaction with PAO does not occlude nucleotide binding. However, the binding site of PAO on Rac1 was not probed as no change in activity was observed upon exposure. Whether PAO-mediated oxidation of RhoA, which prevents nucleotide binding, is related to the function of PAO as a tyrosine phosphatase inhibitor is presently unclear.

In other contexts, a number of studies have demonstrated that ROS/RNS are capable of activating RhoA. For example, we have previously reported in vitro evidence that supports RhoA activation upon oxidation.[44] Treatment of RhoA with nitrogen dioxide induced nucleotide dissociation and subsequent disulfide bond formation between Cys^{16} and Cys.^{20} The disulfide bond occluded nucleotide binding. However, nucleotide association could be observed when the radical scavenging agent ascorbate was present in the reaction. Based on these findings, we speculate that exposure of RhoA to select oxidants can promote intramolecular disulfide formation. However, under normal physiological conditions, the disulfide is likely reduced, which results in guanine nucleotide cycling and activation; under conditions of oxidative stress, disulfide reduction may not occur, thereby promoting RhoA inactivation. Thus, oxidation of RhoA could lead to activation or inactivation depending on the cellular redox state.

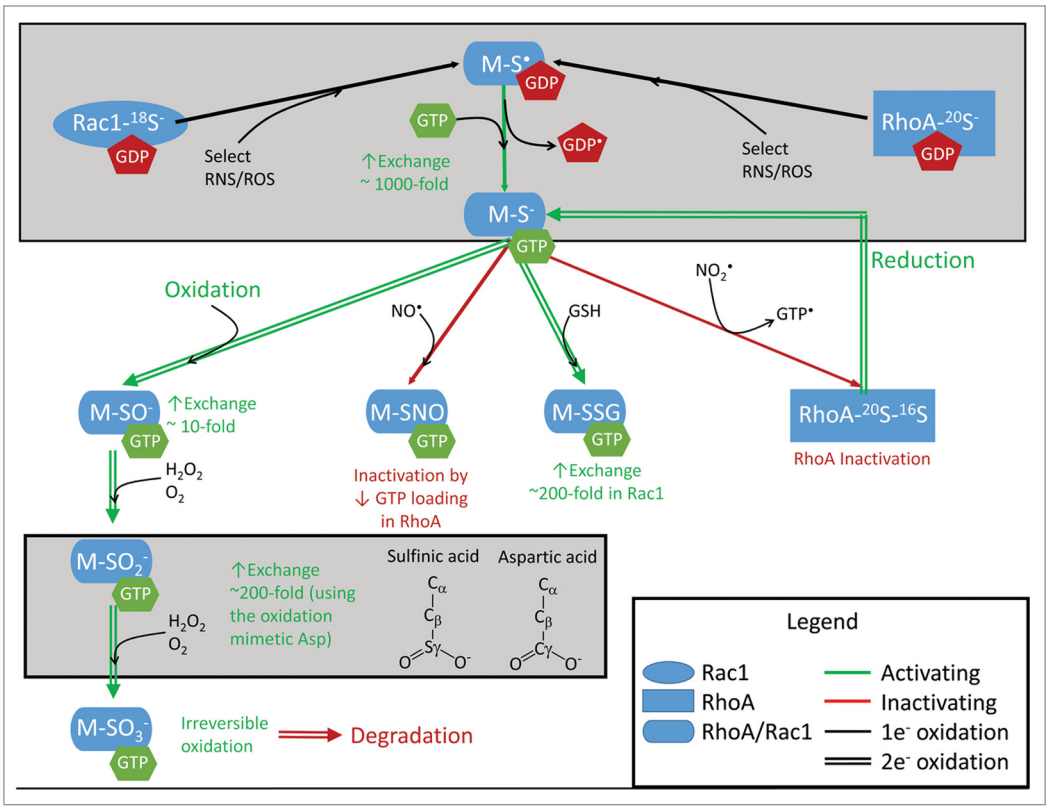


Figure 6. Thiol oxidation of Rac1 and RhoA GTPases and effects on nucleotide binding and exchange. 'M' represents RhoA or Rac1. Rac1 and RhoA have a redox-sensitive thiol at Cys[18] and Cys,[20] respectively. At physiological pH, this is populated in the reactive thiol anion form. Free radical-mediated oxidants, such as $NO_2^{\cdot}$, can promote thiyl radical formation, cause nucleotide oxidation, and enhance the intrinsic rate of nucleotide dissociation by ~1000-fold, leading to activation of Rac1 or RhoA. In addition, Rac1 and RhoA are susceptible to 2-electron oxidation. Oxidation of M-S$^-$ to sulfenic acid by peroxide promotes the intrinsic rate of nucleotide dissociation by ~10-fold. Oxidation to sulfinic acid, approximated by use of a Cys > Asp mutation (Rac1^{C18D} and RhoA^{C20D}), shows greatly increased rates of intrinsic nucleotide dissociation (unpublished results). Further oxidation to sulfonic acid is irreversible and can lead to protein degradation, which likely occurs under oxidative stress conditions. Distinct oxidative modifications can differentially affect the activity of Rac1 and RhoA. RhoA can be inactivated upon reaction with nitric oxide ($NO^{\cdot}$) in cellulo and in vitro. Rac1 glutathiolation greatly increases nucleotide exchange, similar to the Rac1^{C18D} oxidation mimetic. The formation of an intramolecular disulfide bond is unique to RhoA due to the additional presence of Cys[16]; this results in loss of nucleotide binding. However, conditions that promote reduction of the disulfide bond in RhoA can facilitate nucleotide exchange and activation.

In a study on peroxynitrite-mediated diabetes-induced endothelial dysfunction, peroxynitrite was shown to activate RhoA. Either high glucose or exogenous peroxynitrite treatment for 18 h doubled the amount of active RhoA in bovine aortic endothelial cells, as determined by pulldown with GST-RBD (GST-Rhotekin Rho-binding domain) beads.[128] The glucose-induced increase in RhoA-GTP was blocked by FeTTPS (5,10,15,20-tetrakis[4-sulfonatophenyl]porphyrinato iron [III]), which isomerizes peroxynitrite to nitrate. In human breast cancer cell lines, Lopez-Haber and Kazanietz found that the Jak2 inhibitor cucurbitacin I inhibited Rac1 activation but enhanced activation of RhoA and RhoA downstream signaling independent of Jak2. The effects of cucurbitacin I on Rac1 and RhoA were dependent on intracellular ROS generation as treatment with the ROS scavenger NAC ablated the response due to cucurbitacin I exposure.[136] Yu et al. characterized the time course of RhoA activity following H_2O_2 stimulation in MDCK (Madin-Darby canine kidney) cells. They found a ~1.75-fold increase in RhoA-GTP levels that peaked approximately 5 min post stimulation.[137] Likewise, in the human lung adenocarcinoma epithelial cell line A549, H_2O_2 was shown to activate RhoA by ~2.5-fold after 15 min, which was concomitant with stress fiber formation.[138] As the mechanism of RhoA activation was not investigated in any of these studies, it is unclear whether RhoA activity is regulated directly by oxidants or indirectly through the redox-sensitive LMW-PTP, another GAP, GEF, or GDI.

Indeed, the mechanisms by which ROS and RNS regulate RhoA activity are not yet fully understood, and emerging data suggest that they are likely diverse. Thus, the seemingly opposing observations above may be explained by differential redox regulation that varies between cell type and stimuli. Depending on the specific cellular environment, redox agents can either lead to the formation of intramolecular disulfide bonds that prevent guanine nucleotide binding,[44] thereby inactivating RhoA, or can facilitate guanine nucleotide dissociation,[44] thereby activating RhoA due to the higher levels of cellular GTP vs. GDP. In a separate mode of activation, indirect regulation of RhoA by oxidants has been observed as well. A study on arginase activity

in endothelial cells indicated that peroxynitrite and peroxide increased PKC-mediated phosphorylation and activation of p115-RhoGEF, which subsequently increased RhoA-GTP levels.[139] Thus, in at least one context, activation of a RhoGEF can lead to RhoA activation by ROS. Kondrikov et al., however, proposed a different mechanism for ROS-dependent RhoA activation in lung fibroblasts. In their model, it was proposed that release of GDI from RhoA would promote RhoGEF binding and a subsequent increase in RhoA-GTP levels.[140]

In contrast, few studies have determined whether ROS and RNS can act directly on Rho GTPases in cells to activate them through covalent modification of specific residues. Our group has observed direct activation of RhoA by ROS that involves the 2 redox-sensitive cysteine residues (Cys^{16} and Cys^{20}) within the phosphoryl-binding loop of RhoA. By treating REF-52 cells (rat embryonic fibroblasts) with low concentrations of exogenous peroxide, we were able to show increased stress fiber formation and concurrent RhoA-GTP loading, both of which were abolished by mutating the redox-sensitive cysteines to alanine residues.[141] Together, these findings imply that RhoA activation by ROS can be either direct (amino acid residue modification) or indirect (RhoA regulator modulation) depending on the cellular context, or could even be a combination of both, which has not been ruled out in any study.

Peroxide reacts with thiolates at rates dependent on both the pK_a and solvent accessibility of the thiol.[142] As predicted by Winterbourn et al.,[48,143] the rate of peroxide oxidation of thiol-containing proteins is likely too slow to reversibly regulate GTPase activity on the timescale of most physiological events. Therefore, we predict that oxidants capable of reacting with the redox-sensitive p-loop cysteine in Rho GTPases to generate a thiyl radical (i.e., nitrogen dioxide and superoxide) are more likely to alter cellular Rho GTPase activity by promoting nucleotide exchange. As the redox-sensitive cysteine forms direct interactions with the guanine substrate, non-radical oxidation likely alters guanine nucleotide binding and leads to sustained guanine nucleotide exchange and activation (**Fig. 6**).

Conclusions and Future Directions

While the guanine nucleotide binding properties of RhoA and Rac1 can be modulated directly by oxidants in vitro, few cellular studies have been conducted to determine the role of direct oxidation in regulating their activity. Nevertheless, increasing evidence has tied both RhoA and Rac1 to regulation by oxidation in cells. Given the relative reactivity of free radical oxidants compared with 2-electron oxidation, it is likely that free radical oxidation of reactive cysteines in the p-loop of Rho GTPases regulates Rho GTPase activity and signaling in vivo. Under reducing conditions, we speculate that free radical oxidants promote Rac1 and RhoA activation by facilitating nucleotide dissociation and GTP-loading, similar to the function of GEFs. However, under conditions of oxidative stress, these GTPases may become inactivated. The studies highlighted in this review raise numerous questions, several of which require technically challenging approaches to address. For example, as the redox-sensitive thiol in Rho GTPases likely has an altered pK_a, which causes it to be in the thiolate state at physiological pH, the sensitivity of this site to both 1-electron and 2-electron oxidation events is increased relative to other cellular thiols that lack altered pK_a values. Distinct oxidative modifications can result, such as nitrosation, glutathiolation, sulfenic, sulfinic, sulfonic acid, or disulfide formation, and each of these oxidation products may uniquely alter Rho GTPase activity. Therefore, using in vitro assays to elucidate how distinct oxidative byproducts alter Rho GTPase activity using stoichiometric and specific oxidation, as has been done with Ras,[37,38] will prove valuable in interpreting cellular and in vivo data on Rho oxidation. Our preliminary findings on Rac1 suggest that distinct oxidative thiol modifications have different effects on nucleotide binding properties, but verification in vivo is required. To better understand how oxidants directly regulate redox-sensitive Rho GTPases in cellulo or in vivo, the Rho GTPase oxidation state should be determined when possible or redox-insensitive variants should be used. In addition, the vast majority of observations in cells have utilized exogenous oxidants that not only alter the levels of oxidants in a non-physiological manner but also alter the redox state of the cell. Investigation of the cellular redox state with endogenously produced oxidants will be necessary to characterize how Rho GTPases are regulated by oxidation in physiological settings. The report by Aghajanian et al.[141] was one of the first to show the cellular activation of $RhoA^{WT}$ but not of redox-insensitive RhoA variants upon exposure to exogenously and endogenously applied oxidants and currently remains the only study that reports on the direct oxidation of RhoA in cells. This type of study is still scarce-to-nonexistent for other Rho family GTPases. Given the conservation of redox-sensitive cysteines in Rho GTPases, it is highly likely that other Rho family members are also regulated directly by RNS and ROS. Determining the specificity of ROS/RNS actions on Rho proteins, and uncovering the mechanisms behind these events, will be exciting challenges for the foreseeable future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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