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Forum Review

Intracellular Redox Regulation by the Family of Small GTPases

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ABSTRACT

There is growing experimental evidence that the production of intracellular reactive oxygen species (ROS) represents a tightly regulated process. In particular, numerous observations have suggested a role for the Ras superfamily of small GTPases in redox regulation. This article reviews the evidence that ROS can serve as important downstream effectors for both Ras and Rac proteins. Given the prominent role these proteins play in regulating growth, senescence, and transformation, understanding the role of the small GTPase family in redox regulation may significantly alter our current concepts as to how free radicals contribute to diverse processes from aging to cancer. *Antioxid. Redox Signal.* 8, 1857–1863.

INTRODUCTION

The biology of the small GTPase family is an ever-expanding field that touches on virtually every aspect of cellular homeostasis. In general, these proteins are viewed as a family of molecular switches whose activity is regulated by guanine nucleotide binding. In the GTP-bound state, the switch is generally viewed as being "on," while the reverse is true when the protein is bound to GDP. Under basal conditions, the small GTPase are bound primarily to GDP. Upstream activating signals, such as the binding of a cognate ligand to its receptor, stimulates the activity of GTPase exchange factors (GEFs) that catalyze the exchange of GDP for GTP. This switch in guanine nucleotide binding alters the three-dimensional conformation of the GTPase, allowing it now to specifically interact with multiple downstream targets. Initial interest in the small GTPase family was catalyzed by the observation that Ras proteins are commonly mutated in a number of solid tumors (9). Analysis of the activating mutations in these oncogenic forms of Ras revealed that mutations were confined to specific regions of the protein, resulting in structural alterations that locked the protein in the 'on' configuration. Similar structural mutations can be made in almost all members of Ras superfamily, resulting in constitutively active forms of these proteins. Use of these constitutively active or corresponding dominant negative forms of the small GTPases have provided useful tools to assess the role these proteins play in the regulation and production of intracellular reactive oxygen species (ROS).

LESSONS FROM THE NEUTROPHIL

Chronic granulomatous disease (CGD) is an inherited disease characterized by recurrent and sometime fatal infections along with chronic granuloma formation (34, 36). Although rare, affecting approximately 1 in 200,000 individuals, the disease has proved to be instructive to those investigators interested in redox regulation. Early analysis revealed that activated neutrophils and macrophages derived from affected individuals failed to appropriately generate ROS such as superoxide and hydrogen peroxide. Since the production of ROS represents an important aspect of neutrophil host defense, this deficiency in the phagocytic respiratory burst provided an important clue as to why CGD patients suffered from recurrent infections. Subsequent molecular analysis revealed that CGD patients usually have inherited defective alleles in one of a set of four separate proteins that constitute what is now termed the neutrophil NADPH oxidase (13). This complex is thought to include two membrane bound components, initially termed gp91phox and p22phox, as well as two cytosolic elements, p67phox and p47phox. Mutations in each

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of these four components have been described in patients with CGD, though mutations in the X-linked gp91phox subunit remain the most common cause of the disease. The gp91phox subunit has been demonstrated to bind a number of important cofactors required for oxidant generation including FAD, NADPH, as well as heme. Subsequent analysis of the NADPH oxidase revealed one additional important component, namely the small GTPase Rac. The neutrophil expresses two different Rac isoforms, including the phagocyte specific Rac2 and the more ubiquitously expressed Rac1. Although rare, mutations in Rac2 have been described that result in phagocyte immunodeficiencies, arguing for an essential role of this GTPase in human neutrophil biology (2, 48). Detailed molecular analysis beyond the scope of this review has revealed that Rac proteins function as a necessary switch for ROS generation and that the protein is recruited to the membrane following neutrophil activation where it can bind to both p67phox and gp91phox (13).

As discussed above, the production of ROS by the neutrophil is a highly regulated event, triggered by neutrophil activation. In the laboratory, such activation is often induced by the addition of agents such as fMLP or phorbol esters. Thus, at least for the case of phagocytes, specific ligands or intracellular signaling molecules trigger the activation of the NADPH oxidase and the subsequent production of ROS. While this phenomenon of regulated ROS production in response to ligand stimulation was initially viewed as restricted to professional phagocytes, the last decade has seen a significant reappraisal of this assumption. For instance, we demonstrated that in vascular smooth muscle cells, the addition of PDGF produced a rapid rise in intracellular ROS (41). Rather than being required for host defense, this production of ROS appeared necessary for normal mitogenic stimulation. Subsequent studies have extended these observations and have implicated the production of ROS as an essential element of signal transduction in a large number of pathways (10). Evidence suggests that certain aspects of neutrophil biology appear to be conserved in the ROS signaling of nonphagocytic cells. For instance, the expression of a constitutively activate forms of Rac1 was noted to increase the basal level of hydrogen peroxide in immortalized fibroblasts (42), as well as in certain transformed cell lines (40). This increase in ROS could be blocked by the flavin inhibitor diphenylene iodonium (DPI), an agent that also inhibits ROS generation from the neutrophil NADPH oxidase (40, 42). Further experiments demonstrated that in vascular smooth muscle cells, antisense inhibition of p22phox blocked ROS generation following the addition of angiotensin II (45). Similarly, vascular smooth muscle cells derived from mice with a targeted disruption of p47phox demonstrated a significant reduction in ROS production in response to phorbol ester or to ligands such as angiotensin II or PDGF (22). Finally, the expression of a dominant negative form of Rac1 was shown to inhibit the production of ROS following addition of various ligands (42). Taken together, these results suggest that a Rac-regulated oxidase exists in a wide range of cell types and participates in normal signal transduction.

One initially disquieting aspect of the above experiments was the observation that an essential component of the classical NADPH oxidase, the gp91phox subunit, was generally viewed as having a restricted pattern of expression, residing essentially only within phagocytic cells. What then was the molecular composition of the Rac-regulated oxidase in nonphagocytic cells? An explanation for this dilemma came with the cloning of Nox1, an enzyme with significant homology to gp91phox but very different tissue distribution (39). Indeed, Nox1 appears to be expressed primarily in colon, uterus, prostate, and vascular smooth muscle cells. Subsequent analysis has expanded the Nox family to include at least five members with the classical neutrophil gp91phox subunit now commonly referred to as Nox2. All Nox family members share some degree of sequence identity ranging anywhere from 20% to 60%, but more importantly possess common structural motifs including NADPH and FAD binding domains, as well as putative heme binding regions (19). In addition, tissue specific isoforms of both p47phox and p67phox have been recently described (6, 11, 43). The biology of these novel Nox proteins and their intracellular partners is an active area of research and compared to the classical neutrophil NADPH oxidase system, relatively little is known regarding their molecular regulation or their presumed tissue-specific biology. Recently, however, a study demonstrated that Nox1 constitutively binds the Rac guanine exchange factor βPix (32). Growth factor stimulation was demonstrated to cause the binding of Rac1 to Nox1 in an analogous fashion that has been previously described in neutrophils, where agents such as fMLP cause the association of Rac2 with Nox2. This and previous studies (8) also support a pathway where ligand addition results in the sequential activation of phosphatidylinositol 3-kinase (PI3K), which in turn generates lipid products that can activate βPix or other GTPase exchange factors through the pleckstrin homology domain present within the GEF. Activation of the exchange factor in turn leads to increased Rac activity that is presumed to directly stimulate Nox (Fig. 1).

The importance of Rac-regulated ROS generation is underscored by the observations that antioxidants can block many important aspects of signal transduction (10). Early examples of this included a role for ROS in the signaling pathways of PDGF (41), EGF (5), and angiotensin II (44). More recent examples include a role for Rac-regulated ROS in the crosstalk between G protein-coupled receptors (GPCRs) and the JAK/STAT pathway (33). Again, these and other studies have demonstrated that ROS act downstream of Rac as specific biological effector molecules.

Another aspect of oxidant signaling derived from the initial observations that Rac proteins regulate ROS levels in nonphagocytic cells is the recent observation of redox-dependent crosstalk between different GTPase family members. In this case, the authors were investigating the molecular basis for the commonly observed phenomenon that the activity of Rac proteins is often inversely related to the activity of another small GTPase, RhoA. Both of these small GTPases are involved in multiple aspects of cell biology, including an important role in cytoskeletal dynamics. While not universally true, in general, conditions that lead to high levels of Rac activity usually result in low RhoA activity, and vice versa. Interestingly, the reciprocal regulation appears to be dependent on Rac-regulated oxidant production (31). In particular, these authors demonstrated that Rac-stimulated ROS production directly modulated the activity of low molecular weight protein tyrosine phosphatases (LWW-PTP). These PTPs in turn

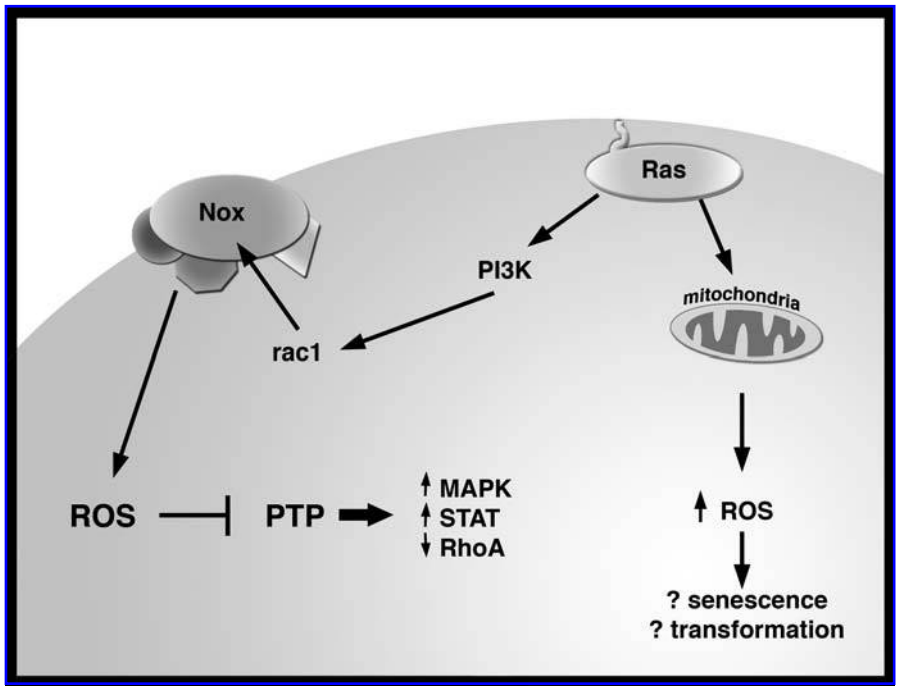

FIG. 1. Regulation of intracellular ROS levels by the small GTPases. Both Ras and Rac proteins are important regulators of the intracellular redox state. Rac proteins regulate the activity of the NOX family of NADPH oxidases, an important source of ligand-stimulated ROS production in both phagocytic and nonphagocytic cells. In some situations, activation of Rac occurs through a Ras-dependent and PI3-K regulated pathway. Increased levels of ROS appear to have many targets within cells, including protein tyrosine phosphatases (PTPs). These PTPs in turn regulate the activity of numerous signaling molecules, including members of the MAPK family, the JAK/STAT family, as well as other small GTPases, such as RhoA. There is also some evidence that Ras proteins may directly or indirectly regulate mitochondrial oxidant production. This increase in mitochondrial ROS production may be important for the well-studied biological properties of Ras including both transformation and senescence. See text for details.

regulate the activity of p190Rho-GAP, which is a normal regulator of RhoA activity. The modification of tyrosine and dual specific phosphatases was initially hypothesized as an important target of ROS that linked a rise in ROS to downstream signaling (41). This study, as well as others (27, 35) has greatly solidified this hypothesis.

Finally, there is one additional example where the knowledge that Rac proteins are important regulators of the intracellular redox state has proved to be useful. For many years it was appreciated that immediately following the reinstitution of oxygenated blood into ischemic tissue, there was a rapid burst of ROS. Blocking the ROS at reperfusion was viewed as an important therapeutic goal since this is a common clinical situation occurring in a variety of surgical procedures, including, for instance, the treatment of myocardial infarctions by emergency angioplasty. The molecular basis and source for the burst of ROS following reoxygenation has remained obscured. Again, *in vitro* experiments have suggested that expression of dominant negative Rac1 can significantly inhibit ROS generation following hypoxia-reperfusion (17). Similarly, in an *in vivo* situation, adenoviral transduction of the liver with a dominant negative Rac1 has been recently demonstrated to suppress ROS generation following ischemia/reperfusion as well as significantly attenuating hepatic injury in this setting (14).

RAS-GENERATED ROS IN TRANSFORMATION AND SENESCENCE

Similar to what was observed with Rac proteins, early experiments demonstrated that in immortalized, nonphagocytic cell lines, transient expression of activated Ras gene resulted in higher levels of intracellular ROS (42). Given that activated Ras can transform immortalized cells such as NIH 3T3 cells, these experiments were consistent with subsequent observations that stable expression of Ras in NIH 3T3 cells produced higher level of ROS and that this oxidant production was important for transformation (16). Similar overexpression of Ras in other cell types such as keratinocytes (52) and epithelial cells (51) also demonstrated an increase in basal ROS levels. The pathway by which Ras regulates the levels of ROS remains incompletely understood. There is some evidence that at least in some cells, it proceeds through a PI3K and Rac-dependent pathway (8) and the subsequent regulation of a cytosolic Nox-dependent oxidase. In other cell types, as will be discussed later, the source of Ras-induced ROS appears to be linked to the mitochondria (23). While these differences may be explainable by differences in experimental design or cell type, there is also some evidence that small GTPases may regulate mitochondrial function in a redox-dependent fashion. In particular, in integrin signaling, both Rac and RhoA appear to regulate the mitochondria, at least in part through the ability of these small GTPases to alter intracellular ROS (47). It therefore remains possible that ROS generated from small GTPases could regulate mitochondrial properties, including the overall metabolic rate and the subsequent generation of mitochondrial oxidants. Subsequent oxidant generation from the mitochondria may also have important signaling functions within the cell that are just now beginning to be explored (3, 7, 30).

The role of Ras-regulated ROS production in cellular transformation has been the subject of several recent interesting studies. First, a recent proteomic study compared two related ovarian cell lines (53). While both cell lines were immortalized by SV40 T/t antigen and telomerase expression, only one

of the cell lines also expressed an activated Ras allele. Of the greater than 2,000 proteins analyzed by two-dimensional gel electrophoresis and mass spectroscopy, the authors identified approximately 30 proteins that showed significant changes between these two ovarian cell lines. Interestingly, the majority of these differentially expressed proteins clustered around proteins involved in either cellular metabolism or antioxidant defense. Again, these results imply that Ras proteins appear to produce a pro-oxidant environment and suggest, but certainly do not prove, that this may be important in the gene product's transforming capacity.

Another recent study showed that Ras expression in certain cell types markedly upregulates the expression of Nox1 (28). Nox1, as discussed previously, is a member of the growing NADPH oxidase family (19). Interestingly, the use of small interfering RNAs directed at Nox1 blocked a number of the Ras-induced transformed phenotypes, including anchorage-independent growth and tumor formation *in vivo*. These results expand on the tumor biology of Nox1 that previously was shown to be involved in tumor angiogenesis in a redox-dependent fashion (4) and argue that in certain cells, Nox1 is an important downstream effector of Ras.

Finally, analyzing cells deficient in junD has provided another important connection between Ras, ROS, and tumorgenesis. JunD belongs to the AP-1 family of transcription factors, and previous observations had suggested that the gene product is involved in limiting tumor formation (26). In a recent study, the use of junD-/- embryonic fibroblasts revealed that the transcription factor was involved in regulating antioxidant defenses and particularly was required to limit Ras-induced ROS (12). In the absence of junD, intracellular hydrogen peroxide levels rose, and the authors demonstrated a subsequent redox-dependent activation of the hypoxia inducible factor Hif-1α. Activation of Hif-1α led in turn to the production of the potent angiogenesis stimulator, VEGF. Therefore, the authors were able to demonstrate that junD expression prevented Ras-induced tumor angiogenesis by limiting ROS levels and hence VEGF secretion.

Whereas the expression of Ras in the context of immortalized cells results in transformation, the phenotype of Ras expression in normal diploid cells is quite different. Indeed, experiments with primary mouse or human fibroblasts revealed that expression of activated Ras in these normal cells resulted in a rapid inhibition of proliferation and the induction of senescence (37). Subsequent experiments have demonstrated that in this context, Ras expression leads to an increase in overall ROS and appears in particular to increase the level of mitochondrial-derived ROS. Scavenging mitochondrial oxidants with *N*-acetylcysteine, or lowering ambient oxygen to 3% (rather than the standard 20%), resulted in an inhibition of Ras-induced senescence (23). In this context, ROS appear to function as mediators or downstream effectors of Ras-induced senescence. A similar rise in ROS appears to accompany other genetic strategies that induce senescence, including the expression of p53 (24), the p53-regulated cell cycle inhibitor p21 (25), or Akt (29). In the context of Ras overexpression, one important downstream mediator appears to be induction of p53 (37). Relatively little is known how Ras activation and subsequent ROS generation leads to an increase in p53 levels. A recent study has suggested, however, that the gene product seladin-1 may be an important intermediary (50). This gene product is an FAD-oxidoreductase previously implicated in cholesterol synthesis (46). Knockdown of seladin-1 using RNA interference appeared to prevent Ras-induced senescence without altering ROS levels, suggesting that the protein was downstream of oxidants but upstream of p53 (50).

Since Ras is a well-conserved protein, it is of interest that several examples have recently emerged demonstrating a role for Ras proteins in redox regulation of simple organisms. For instance, in yeast, expression of an activated form of the Ras2 allele results in an increase in overall ROS (15). This phenotype appears to result from an effect of Ras2 on mitochondrial function. In particular, yeast expressing this activated Ras allele appears to have an altered mode of aerobic respiration leading to a state of high mitochondrial membrane potential and increased ROS production (15). Interestingly, expression of this allele also results in a decrease in the replicative life span of yeast. As such, these results are reminiscent of the observation made in normal fibroblasts where activated Ras expression appears to shorten *in vitro* life span through the generation of mitochondrial oxidants (23). Similarly, in *Caenorhabditis elegans*, it has been appreciated for some time that Ras proteins are involved in normal vulval development. In a recent study however, it was demonstrated that in the worm, Ras-generated oxidants acted as requisite signaling molecule necessary for proper development of this reproductive organ (38). Again, these results suggest that Ras-induced ROS formation is a highly conserved property of this family of proteins and plays an important role as a downstream effector in a number of important contexts.

Although the above studies implicate Ras in the production and regulation of intracellular ROS, there is also abundant evidence that Ras proteins can also be a direct target of ROS. In particular, cysteine 118 of the protein has been shown to undergo redox-modification. The first example of this involved nitric oxide (NO) dependent signaling. Early experiments demonstrated that NO donors were capable of forming a nitrosothiol derivative with this reactive cysteine (20, 21). This modified form of Ras was shown to possess increased guanine nucleotide exchange activity. Subsequent NMR studies have revealed that increased activity was not a result of a change in Ras structure or GTPase activating (GAP) or exchange (GEF) function (49). The same residue (Cys-118) can also be *S*-glutathiolated. This posttranslational modification involves the addition of the tripeptide glutathione onto proteins. Glutathione exists within cells at millimolar concentrations and therefore serves as an important redox buffer. The addition of glutathione to proteins to produce a mixed protein disulfide is a poorly understood process, but one that is gaining considerable attention as a potentially important and specific redox modification (18). In vascular smooth muscle cells stimulated with angiotensin II, the level of glutathiolated Ras proteins was significantly increased (1). This modification appeared to be important for maximal activation of downstream targets such as Akt and p38. Similarly, the glutathiolation of Ras following angiotensin II stimulation could be blocked by various antioxidant proteins, including both catalase and glutaredoxin.

SUMMARY

Although once viewed as an essentially unregulated process, there is a growing appreciation that the production of intracellular ROS is tightly regulated and that oxidants serve as intracellular signaling molecules (10). The precise means of regulation remains incompletely understood. Here we have reviewed the evidence that the family of small GTPases belonging to the Ras superfamily appear to regulate ROS production. This phenomenon is well conserved throughout evolution, as Ras proteins in yeast, *C. elegans,* and mammalian systems appear to function, at least in part, in a redox-dependent fashion. More importantly, the production of ROS by the small GTPases appears to be an important aspect of the function of these molecular switches. Again, this appears to be the case from vulval development in the nematode (38) to the ability of mammalian Ras to induce either transformation or senescence (16, 23). Similarly, the coordinate regulation of the small GTPases such as the functional interplay between Rac and RhoA activity appears to have an important redox-dependent component (31). Taken together, these data collectively argue that oxidants function as effector molecules for the small GTPases, and therefore contribute to their overall biological function. Given the extensive role this family of proteins plays in multiple biological contexts, these observations may have important implications as to the mechanism through which oxidants contribute to both human health and disease.

ABBREVIATIONS

CGD, chronic granulomatous disease; DPI, diphenylene iodonium; EGF, epidermal growth factor; fLMP, *N*-formyl-Met-Leu-Phe; GEF, GTPase exchange factor; GPCR, G-protein coupled receptor; PDGF, platelet derived growth factor; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor.

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