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Stress, Superoxide, and Signal Transduction

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A variety of stressful events can trigger the production of free radicals by exposed cells. For years, the effect of such highly reactive radicals was expected to be damaging to cells, altering their biology irreversibly. However, many recent reports have shown that reactive oxygen species can have additional functions, and contribute to important signaling pathways to regulate key biological responses, including cell migration, mitosis, and apoptosis. With this review, we address the role of the small GTP binding protein, Rac, as a regulatory protein that controls superoxide production, and the effect of superoxide and derived oxidants in cell signaling.

Stress Reactive oxygen species Superoxide Rac Small GTP binding proteins
Signal transduction Actin Review

WHEN the endothelium of blood vessels becomes injured, either mechanically, due to a loss of shear (39) or to coronary angioplasty, or chemically by oxidized low-density lipoproteins in patients with elevated circulating cholesterol, the rate of apoptosis of endothelial cells (EC) can be markedly accelerated (11,40). Consequently, the surface of the vessel wall can be depleted of EC, and adjacent EC are triggered to migrate and proliferate to reconstitute a confluent monolayer. We have recently observed that such a response to injury, or stress response, is accompanied by the production of heightened amounts of reactive oxygen species (ROS), and in particular superoxide, by the endothelial cells (32).

Previously, the superoxide anion had been associated with the bactericidal activity of phagocytes. A major source for superoxide in cells consists of the enzymatic complex: nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Bactericidal superoxide is released within phagolysosomes where microorganisms are being degraded. The switch for the activation of NADPH oxidase is provided by Rac, a small GTP triphosphatase protein of the Ras/Rho family of proteins (16). Although it may appear paradoxical that superoxide be involved in both cellular injury and tissue repair, there are precedents for such a dual role played by oxidants. In the nitric oxide (NO) system, low concentrations of NO transduce signals within vessels and neurons, whereas high concentrations of NO can damage cells and microorganisms. By analogy, superoxide, and probably other oxidants, serve as messengers when present at low concentration, while larger amounts are required for cell killing. It is conceivable that, throughout evolution, the role of superoxide has progressed from being a purely damaging radical to becoming a mediator for sophisticated responses such as cell migration or liquid phase pinocytosis.

REGULATION OF Rac BY ITS BOUND GUANYL NUCLEOTIDE

The small GTP triphosphatases form a large family of proteins (Ras family) whose activity is regulated by the binding, hydrolysis, and release of guanosine trisphosphate (2,12,16). Rac binds GTP with a K_d that is several orders of magnitude smaller than the concentration of guanyl nucleotides in cells. Therefore, Rac activity is not controlled by the cellular level of GTP (3,12). Instead, Rac's interaction with GTP is regulated by protein ligands, which modulate its interaction with the bound nucleotide (2,3,12,16). For example, in quiescent cells, Rac is found as a complex with GDP and a protein ligand that inhibits the exchange of the GDP for GTP (GDP dissociation inhibitor or GDI) (2). When a receptor tyrosine kinase is activated by its extracellular ligand, the GDI dissociates from Rac. Next, GDP-Rac releases its bound nucleotide, a reaction that can be ac-

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celerated by a nucleotide exchange factor. Hence, the nucleotide-free Rac resets for interaction with another guanosine nucleotide. Considering the vast excess of GTP over GDP found in most cells, the nucleotide exchange results in the recharging of Rac with GTP, switching Rac activity back on. Rac activity is also known to require posttranslational modification (41): the sequential methylation and prenylation of the C-terminus of Rac. Such modifications are required for Rac association with the cell membrane. What has remained less well characterized are the effector pathways downstream from Rac that are responsible for its function(s) in cells.

RESPIRATORY BURST IN PHAGOCYTES: A STRESS RESPONSE

In phagocytes, GTP-bound Rac stabilizes the assembly of several proteins, to form an enzymatic complex known as NADPH oxidase (10,26). In the presence of the cofactors, NADPH and flavin adenine dinucleotide (FAD), NADPH oxidase catalyzes the generation of superoxide (8,10,35). The enzymatic activity is provided by a flavocytochrome, cytochrome b558, an integral membrane protein composed of two subunits: glycoprotein (gp) 91phox and p22phox. The enzyme catalyzes the following reaction: $NADPH + 2O_2 \rightarrow 2(^{\bullet}O_2^{-}) + NADP + H^{+}$. The activity of the b558 is dependent upon its interaction with additional components of the complex: p67phox, p47phox, p40phox, and Rac. These subunits are cytoplasmic in resting phagocytes, but join b558 at the membrane upon activation of the respiratory burst (8,35). They cluster through the interaction of src homology domain-3 (SH_3) modular elements with domains rich in proline (9).

The timing for the activity of this multimolecular complex is controlled through the hydrolysis rate of the GTP bound to Rac. Two Rac isoforms have been identified in mammals: Rac1 and Rac2. Rac2 has a

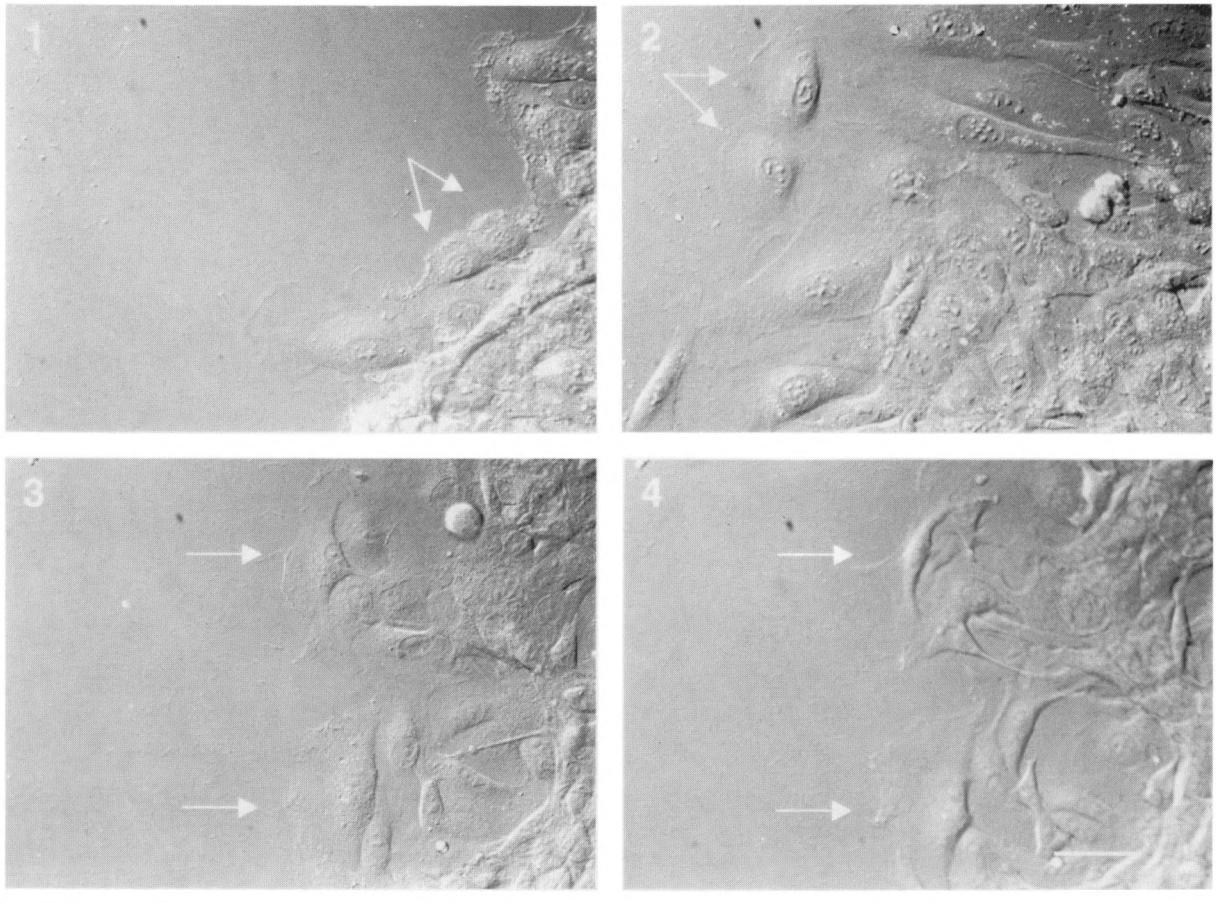

FIG. 1. Cell motility: a response to stress. A superoxide scavenger (the superoxide dismutase mimetic MnTMPyP) inhibits mouse endothelial cell migration. Confluent monolayers were "wounded" with a pipet tip, and the cell migration into the wound was followed by time-lapse microscopy for 5 h. 1 and 3: time zero; 2 and 4: same fields, after 5 h. 1 and 2: untreated cells; 3 and 4: migration in the presence of 25 μM MnTMPyP. Arrows point towards two cells in each experiment, which were followed during the 5-h migration. The bar is 50 μm.

higher affinity for the NADPH oxidase than Rac1, and seems to be constitutively associated with membranes, whereas Rac1 translocates from the cytosol to the membrane together with the other b558 ligands, upon stimulation of the respiratory burst (27,28). It is possible that Rac2 represents a specialized Rac isoform designed to induce the production of bactericidal concentrations of superoxide anion, whereas Rac1 could be involved in the generation of smaller concentrations of superoxide. In support of this concept, Rac2 represents ≥ 95% of total Rac in neutrophils, a major superoxide-producing phagocyte, and depends only on b558 for its interaction with membranes. In contrast, Rac1 depends upon its interaction with p67phox to activate the NADPH oxidase (27). Several domains of Rac seem to be implicated in activation of NADPH oxidase, in particular a highly conserved N-terminal effector region (G1), and other discrete regions dispersed across the protein, including the key residues histidine 103 and lysine 166 (human Rac numbering) (13,14,25,33). Recently, several groups have reported that Rac activation of NADPH oxidase is not limited to phagocytes (19,23,24,37,42,44,47).

Some effects of Rac depend on the activity of downstream kinases, in particular PAK65 and POR (16,29). However, some effects of Rac are independent of these kinases, and the effectors for the several essential non-kinase-mediated activities of Rac have remained uncharacterized. A landmark of cell transformation by oncogenic *ras* consists of the ability of the transformed cells to proliferate even in conditions of restricted supply of growth factors and nutrients. We have shown that H-Ras^{V12}-transformed NIH 3T3 cells produce superoxide constitutively (19). Moreover, we have provided evidence that the unchecked proliferation of H-Ras^{V12}-transformed cells in serum-deprived conditions was inhibited by exposure of these cells to a cell permeant antioxidant (*N*-acetylcysteine), in a concentration-dependent fashion (19). Thus, proliferation of these cells was directly dependent upon the concentration of superoxide. Moreover, overexpression of a dominant negative isoform of Rac1 (Rac1^{N17}) inhibited both superoxide generation and unchecked proliferation, placing Rac and its ability to induce the production of superoxide at the center of the transformed character of H-Ras^{V12} cancer cells. Our observation that superoxide induction by Rac in transformed cells is key to the unchecked proliferation of such cells has been reproduced in other laboratories (25). In contrast, NIH 3T3 fibroblasts transformed with a constitutively activated isoform of the serine and threonine kinase Raf, an effector kinase for Ras that results in the activation of MAP-kinase (4,30,31,45), produced much lower amounts of superoxide, and were not significantly inhibited in their proliferation by antioxidants (19).

Several mechanisms could explain the contribution of superoxide to transformation by *ras*^{V12}. The targeted damage of chromosomal DNA (or altered repair), leading to an enhanced rate of oncogenic mutations, or to a loss of tumor suppressor gene products (6,15,22), has been proposed as a key mechanism for the loss of mitogenic control. ROS could also promote the activation of nuclear factor-κB (NF-κB), a transcription factor whose activation has been linked to Ras-mediated inhibition of apoptosis induced by cytokines like tumor necrosis factor-α (TNF-α) (1,42,46). Hence, superoxide, and possibly other ROS, might contribute to heightened proliferation through inhibition of apoptosis. Although in mammalian cells there is no evidence for proteins capable of "sensing" oxidants (as can be found in prokaryotes) (5,18), there is growing evidence supporting the con-

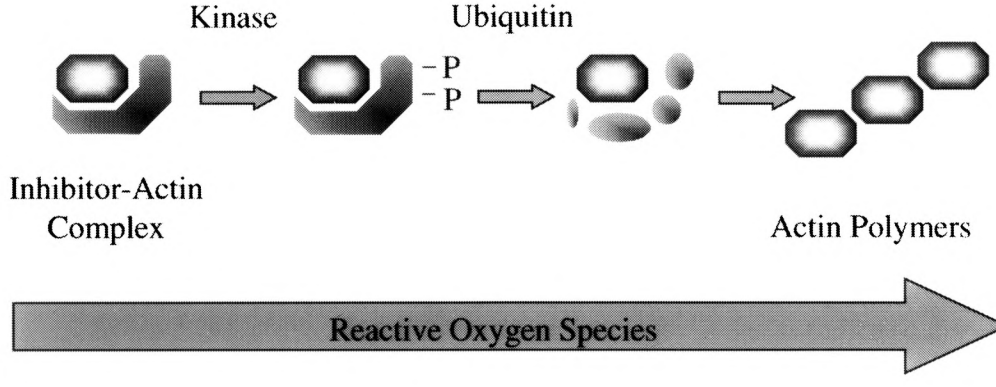

FIG. 2. A hypothetical mechanism for the effect of reactive oxygen species on the organization of the actin cytoskeleton, based on the example of nuclear factor-κB. Actin monomers in the cytoplasm are sequestered away from filaments by an actin monomer binding protein. Upon exposure of the complex to ROS, the inhibitor becomes phosphorylated and then degraded. The actin monomers released by the process can then polymerize.

cept that many signal-transducing proteins and transcription factors are highly sensitive to the redox state of the cells (20,43).

ACTIN CYTOSKELETON REORGANIZATION: ANOTHER RESPONSE TO STRESS

Rac is a strong regulator for the organization of the actin cytoskeleton (16,34,38). Thus far, the intermediates between Rac and the proteins of the actin cytoskeleton have remained only partially characterized (17,25,36). We have studied the role of superoxide (and other ROS) generation in the actin cytoskeleton response to stresses (7,32). As indicated in the Introduction, we have found that endothelial cells involved in the process of repair of a wounded endothelium produce heightened amounts of oxidants (32). Moreover, we found that the cells that produced larger amounts of radicals were also the fastest moving cells, and that the efficient migration of endothelial cells was inhibitable by superoxide dismutase (Fig. 1). Actin polymerization in the migrating cells, as measured by the cytochalasin D-inhibitable incorporation of Alexa-actin into filaments, could be entirely blocked with diphenylene iodonium (DPI). Overexpression in endothelial cells of a constitutively activated isoform of Rac1 ($Rac1^{V12}$), using a replication incompetent adenoviral vector, resulted in the polymerization of actin in a fashion that could be blocked by overexpression of superoxide dismutase. Thus far, our observation in endothelial cells has not yet been reproduced in other cells (25).

Such effects could be mediated, either directly or indirectly, by interaction of ROS with the proteins of the actin cytoskeleton. By analogy with the NF-κB system, oxidants could induce the degradation of proteins that inhibit the interaction of actin with proteins that increase the migratory activity of endothelial cells (Fig. 2). Alternatively, superoxide and other ROS could affect directly the activity of proteins that belong to signal transduction pathways known to regulate the actin cytoskeleton, such as enzymes in the inositol phospholipid or prostaglandin pathways (21,36). The turnover rate of actin filaments in migrating cells can be remarkably rapid. There is simply no mechanism to account for the depolymerization of actin filaments that takes place in such cells at a rate orders of magnitude faster than what has been observed in vitro. It is likely that the altered redox state of migrating cells could contribute to the destabilization of certain actin polymers, through a direct effect of oxidants on actin itself, or on actin binding proteins. We have also found that the actin response to oxidants is exquisitely dependent upon the specific type of oxidant (or antioxidant) that the endothelial

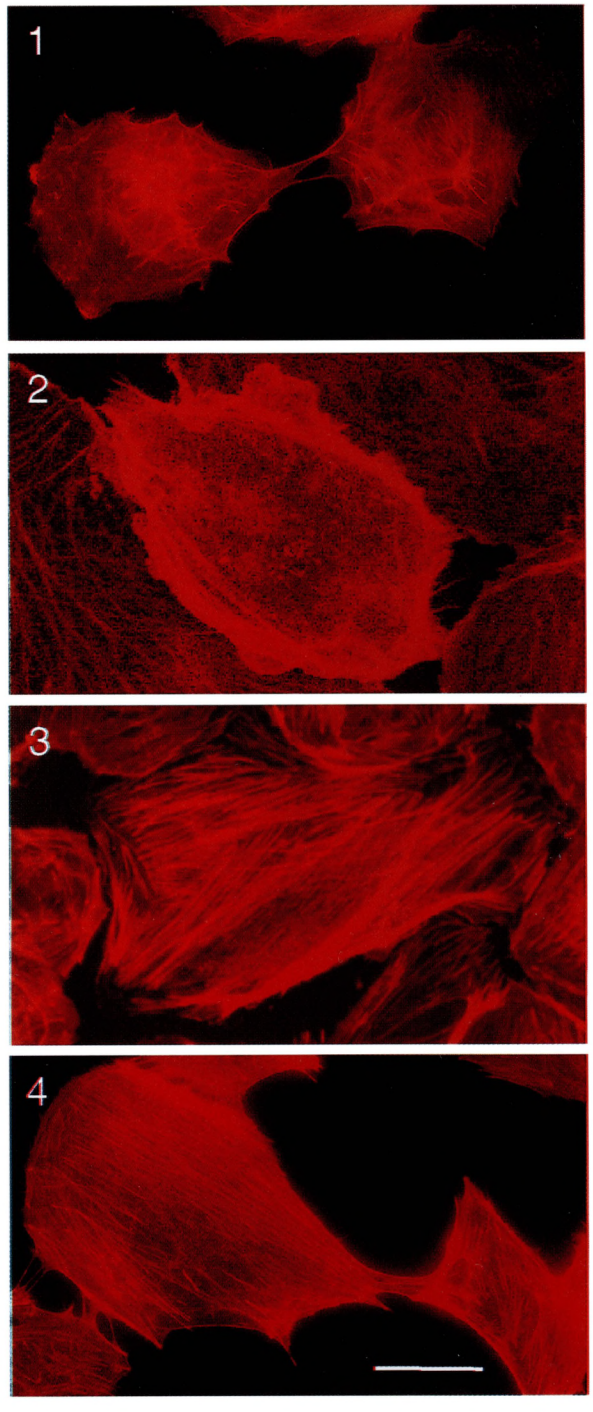

FIG. 3. The presence or absence of different reactive oxygen species has dramatic effect on the actin cytoskeleton organization. F-actin was detected by rhodamine-phalloidin staining in normal human endothelial cells (1), in endothelial cells that overexpress the constitutively active mutant of Rac1 (2), in endothelial cells overexpressing the same Rac1 mutant, but incubated for 1 h with MnT-MPyP (3), and in human endothelial cells treated for 15 min with 100 μM H_2O_2 (4). Note the dissolution of the stress fibers and the intense membrane ruffling in (2), as opposed to the formation of strong actin cables in (3) and (4). The bar is 50 μm.

cells are exposed to (Fig. 3). Such specificity might be due to the physicochemical properties of various oxidants, such as charge, water solubility, and other properties.

CONCLUSION

The response of cells to various stresses includes the production of oxidants and, in particular, superoxide by NADPH oxidase. The precise orchestration of the targeted production of oxygen radicals at specific sites of cells is likely to be timed by the hydrolysis of GTP, bound to the small GTP triphosphatase Rac. In the endless competition among cells, whether prokaryotes or eukaryotes, for nutrients and other resources, one can speculate that the function of free radical production has evolved over millions of years. At first, the generation of free radicals might have been used to eliminate competitors, for example, through destruction of the membrane integrity of competing cells. However, the production of oxidants might have been incorporated progressively into more sophisticated responses to stress, such as, for example, promoting the polymerization of actin as a way to protect the actin monomers from the damaging effect of oxidants (7). Subsequently, such self-defense mechanism might have been incorporated into more elaborated responses, such as the reorganization of the actin cytoskeleton that is necessary for cell migration. Although such a concept remains entirely hypothetical, it was interesting to observe that the speed of moving cells appears to be proportional to the rate of production of oxidants by these cells.

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