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COMMENTARY

Ras, Superoxide and Signal Transduction

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ABSTRACT. The superoxide anion has been associated with the bactericidal activity of phagocytes. Produced by an enzymatic complex, NADPH oxidase, bactericidal superoxide is released within phagolysosomes where bacteria are being degraded. The activity of NADPH oxidase is regulated by Rac, a small GTP binding protein of the Ras family. Recent evidence indicates that, in addition to its bactericidal activity, superoxide seems to function as a signal-transduction messenger, mediating the downstream effects of Ras and Rac in nonphagocytic cells. As such, superoxide contributes to the unchecked proliferation of Ras-transformed cells. In the nitric oxide (NO) system, low concentrations of NO transduce signals within vessels and neurons, while high concentrations of NO can produce damage to cells and microorganisms. By analogy, superoxide and probably other oxidants serve as messengers at low concentrations, while larger amounts are required for inducing damage. The activity of oxidants as messengers opens new avenues for pharmacological intervention against Ras-mediated pathways in mammalian cells. BIOCHEM PHARMACOL **55**;9:1339–1346, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. reactive oxygen species; hydrogen peroxide; superoxide; Ras; small GTP binding proteins; signal transduction; cellular oncogenes; review

REGULATION OF RAS BY ITS BOUND GUANYL NUCLEOTIDE

Small GTP§-binding proteins form a large family of nucleotide triphosphatases whose activity is regulated by the binding, hydrolysis, and release of guanosine trisphosphate (Fig. 1) [1, 2]. A prototype for this family is p21ras (Ras), a protein encoded for by the cellular proto-oncogene *ras* [3–5]. Ras binds GTP with a K_d several orders of magnitude smaller than the concentration of guanyl nucleotides in cells. Therefore, Ras activity is not controlled by the cellular level of GTP. Instead, Ras interaction with GTP is regulated by protein-ligands that modulate Ras interaction with its bound nucleotide [6, 7]. In reconstituted systems, Ras binds to its nucleotide with high affinity and displays weak triphosphatase activity [8, 9]. GAP, accelerates the hydrolysis of GTP by Ras (switch off effect), and exchanger molecules lower Ras affinity for its bound nucleotide, usually GDP (switch on effect). Hence, upon binding to an exchanger protein ligand, Ras disassociates from GDP and, as a result, resets for interaction with another guanosine nucleotide, usually GTP, considering the vast excess of GTP over GDP found in most cells. As such, Ras recharged with GTP is ready for action. Also important is the fact that Ras activity requires post-translational modification [10]. Sequential methylation and isoprenylation (transfer of a farnesyl group) of the C-terminus of Ras are catalyzed by the enzymes methyl-protein transferase and farnesyl-protein transferase, respectively. Such modifications are required for Ras association with the cell membrane, a prerequisite for Ras transforming activity [11]. Hence, the molecular regulation of Ras has been well characterized, including information on its structure at the atomic level [12]. However, for many years, the downstream effector pathways of Ras have remained an enigma.

DOWNSTREAM EFFECTOR PATHWAYS FOR RAS

In *Saccharomyces cerevisiae*, Ras seems to command through at least two pathways. One such pathway, which is unique to yeast, involves Ras interaction with an adenylyl CAP [13]. CAP interacts with adenylyl cyclase, inducing cyclic AMP (cAMP) production. In turn, cAMP regulates growth properties of yeast cells. The activity of CAP appears to involve two separate domains of the protein [14]. While the N-terminus of CAP is responsible for activating adenylyl cyclase, and thereby mediates some effects of Ras that contribute to growth properties, the C-terminus of CAP is involved in the organization of the actin superstructure of these cells. In mammalian cells, Ras does not activate adenylyl cyclase. However, Ras has been shown to switch on and promote the activity of Raf, a kinase which, in turn, triggers the activation of MAP-K (or ERK, through threo-

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§ *Abbreviations:* CAP, cyclase associated protein; CGD, chronic granulomatous disease; GAP, GTPase activating protein; MAP-K, mitogen-activating protein kinase; and ROS, reactive oxygen species.

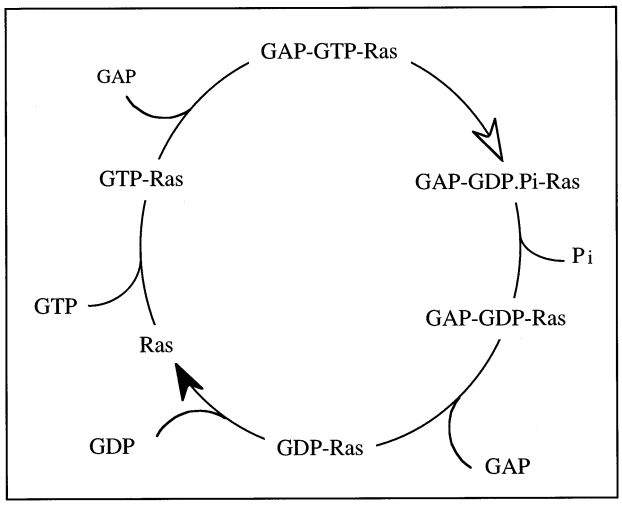

FIG. 1. Nucleotide triphosphatase cycle of Ras. The open arrow indicates the irreversible step for the cycle. The solid arrow corresponds to the reaction that is accelerated by the exchanger molecules. Ras bound to GTP represents the activated conformation of Ras. Abbreviations: P_i, inorganic phosphate; and GAP, GTPase-activating protein.

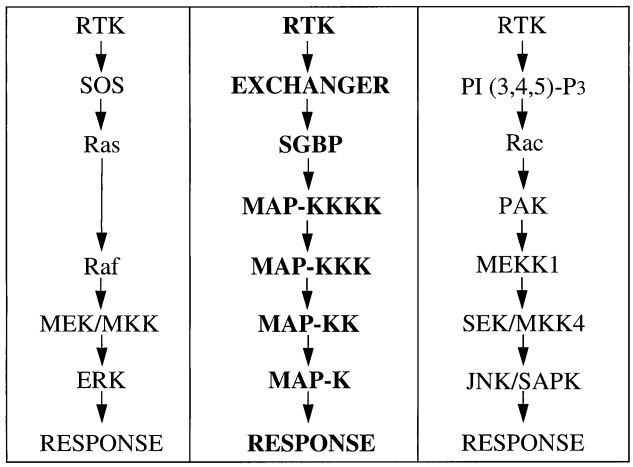

FIG. 2. MAP-K cascade as an effector pathway for activated Ras. A generic cascade (in bold) showing mitogen-activating protein kinases linked in series and transducing signals mediated by small GTP binding proteins (SGBP), under the control of a receptor tyrosine kinase (RTK), which induces the exchange of the nucleotide bound to the SGBP. The successive-Ks-following MAP-K indicate the activity of kinases upstream from MAP-K. Such a cascade has been characterized in the case of Ras and also Rac, or Cdc42 (not shown). SOS is an exchanger protein discovered in drosophila (Son of Sevenless). MEK (or MKK for MAP-KK) is the mitogen and extracellular-regulated kinase; ERK corresponds to extracellular-regulated kinase. PI(3,4,5)-P_3 is a putative exchanger molecule for Rac, and is produced by phosphatidylinositol 3-kinase (PI3-K). PAK is a p21-activated, serine and threonine kinase, PAK65. SEK (or MKK4) is the stress-activated protein kinase (SAPK) activator, SEK-1. JNK is the c-Jun N-terminal kinase.

nine and tyrosine phosphorylation [15–17]. The phosphorylated MAP-K needs to reach the nucleus to modulate the activity of transcription factors, and thereby, in some instances, cell proliferation (Fig. 2).

RAC, SUPEROXIDE EFFECTOR PATHWAY, AND RESPIRATORY BURST IN PHAGOCYTES

While such a kinase cascade represents an important downstream effector pathway for Ras in most cells, evidence has accumulated over the past few years that, as it is the case for yeast, other downstream effectors may be involved, as well, to account for the full effect of Ras [18]. One alternative effector pathway appears to involve another small GTP-binding protein of the Rho family, Rac [19, 20]. In phagocytes, Rac was shown to stabilize the assembly of several proteins, to form a protein structure known as NADPH oxidase [21, 22]. Thus, upon exchanging GDP for GTP, Rac triggers the clustering of a multiprotein complex, which in the presence of the cofactor, NADPH, catalyzes the generation of superoxide [23–25]. The enzymatic activity is provided by a flavocytochrome, cytochrome b_{558}, an integral membrane protein composed of two subunits: glycoprotein (gp) 91phox and p22phox (Fig. 3). The enzyme requires FAD as a cofactor, and catalyzes the following reaction: NADPH + $2O_2 \rightarrow 2 \cdot O_2^-$ + NADP + H^+. The activity of the b_{558} 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 b_{558} at the membrane upon activation of the respiratory burst [26]. They cluster through the interaction of src homology domain-3 (SH_3) modular elements with domains rich in poly-L-proline [27].

Two isoforms of Rac, Rac1 and Rac2, once bound to GTP, promote the assembly of the NADPH oxidase and time the stability of this multi-molecular complex through the hydrolysis rate of the bound GTP [28–30]. Rac2 has a higher affinity for the NADPH oxidase than Rac1, and seems to be constitutively associated with membranes, while Rac1 shifts from the cytosol to the membrane together with the other b_{558} ligands, upon stimulation of the respiratory burst. 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 amounts of superoxide. In support of this concept, Rac2 represents ≥95% of total Rac in neutrophils, the major superoxide producing phagocyte, and depends only on b_{558} for its interaction with membranes. In contrast, Rac1 depends upon its interaction with p67phox to activate the NADPH oxidase, and mutations of p67phox that suppress this interaction mediate rare forms of CGD [31, 32].

CGD is a group of inherited disorders characterized by an extreme susceptibility to pyogenic infections, potentially fatal, believed to result from single mutations of the genes coding for the components of the NADPH oxidase [33–35]. The patients, usually infants or children with cutaneous abscesses and other bacterial or fungal pyogenic infections, are lacking phagocytes with efficient bactericidal activity,

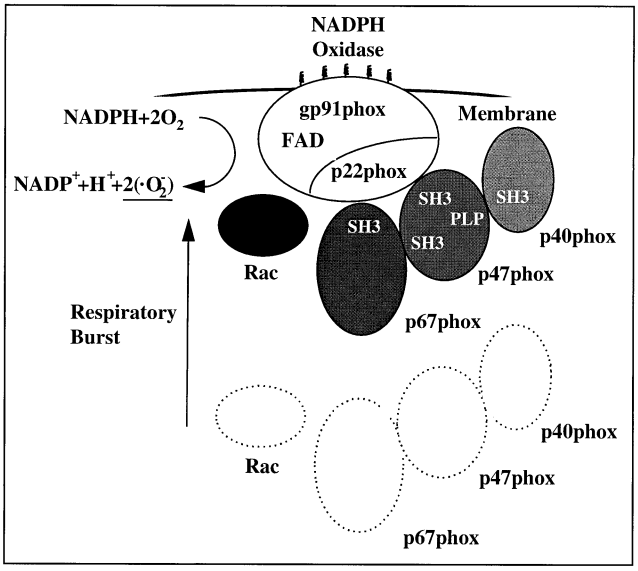

FIG. 3. NADPH oxidase and respiratory burst. NADPH oxidase is a multi-molecular enzymatic complex that produces superoxide anion. A membrane glycoprotein, gp91phox, and its associated subunit, p22phox, compose the flavocytochrome b_{558}. The oxidase catalyzes the formation of superoxide from molecular oxygen and NADPH, in an FAD-dependent fashion. Upon activation of the respiratory burst in neutrophils, the NADPH oxidase is switched on by its interaction with several cytoplasmic proteins: p67phox, p47phox, and p40phox. Clustering of proteins in this complex is mediated by the interaction of modular units, SH_3, with domains rich in proline residues. The stability of this complex is controlled and timed by GTP-Rac and the hydrolysis of the bound GTP.

due to the absence of respiratory burst. For two-thirds of the patients, the defect is in the X-linked gene encoding gp91phox. About 30% of CGD cases are induced by autosomally inherited defects in the p47phox subunit of the NADPH oxidase. The remaining patients have mutations in p22phox and the other components of the complex. A CGD-like transgenic model was reproduced in the mouse by targeted disruption of the gene for gp91phox or for p47phox [36, 37]. Much effort has been committed to the development of gene therapy strategies for the management of patients with this disorder. Virus-mediated gene transfer has been used to restore NADPH activity in phagocytes from patients with CGD, and CGD is a likely major gene disease to be successfully treated with gene transfer in the foreseeable future [38–42].

RAC AND ROS PRODUCTION IN NONPHAGOCYTIC CELLS

Interestingly, fibroblasts from patients with established CGD are capable of producing superoxide, through a flavoprotein-dependent enzymatic system (perhaps an NADPH oxidase complex) [43]. Such data support the concept that more than one NADPH oxidase system contribute to superoxide production in the body: one system is responsible for the bactericidal activity of phagocytes, while at least one other NADPH oxidase complex is responsible for the production of superoxide in nonphagocytic cells. Recently, several groups have reported that Rac activation of NADPH oxidase is not limited to phagocytes [44–47]. Although the specific structure of the putative nonphagocytic NADPH oxidase(s) has yet to be characterized, and some of the protein subunits of this enzymatic activity have yet to be cloned, it seems at least functionally similar to the phagocyte complex [46]. Like Ras, Rac seems to have effects that depend on the activity of downstream kinases (Fig. 2), in particular PAK65 and c-JUN N-terminal kinase (JNK) [48, 49]. However, some effects of Rac are independent of these kinases, and the effectors for the several essential nonkinase-mediated activities of Rac have remained uncharacterized [50, 51].

RAS-MUTATIONS, CELL TRANSFORMATION, SUPEROXIDE PRODUCTION, AND SIGNAL TRANSDUCTION

The *ras* genes are subject to mutations that modulate the interaction of their protein product with GTP [11]. In particular, mutations of the *ras* gene can lead to diminished triphosphatase activity, locking Ras in a "switched on" state. Such mutations have been found in >50% of colon and >90% of pancreatic cancers [52]. Alternatively, mutations can also lead to the very rapid exchange of the bound nucleotide, which, in cells containing a vast excess of GTP over GDP (most cells), would result in the constitutive association of Ras with GTP, with practically no "off-time" where Ras is bound to GDP. Whatever the mechanism involved, mutations that lead to the constitutive association of Ras with GTP are believed to represent an important step in the progression of cells towards unchecked proliferation [53]. However, the specific molecular steps involving mutated Ras that contribute to such cell transformation have remained uncharacterized.

The constitutive production of superoxide could be readily detected in fibroblastic clonal lines (NIH 3T3) stably transfected with a cDNA coding for one such isoform of Ras incapable of hydrolyzing GTP, H-Ras V12 [54, 55]. It was shown that superoxide production in transformed cells was dependent upon farnesylation of H-Ras V12, required Rac1 activity and the activity of a flavoprotein, probably NADPH oxidase, but was independent of mitochondrial oxidases.

Superoxide production was associated with phenotypic changes in these transformed cells [54]. A landmark of Ras-transformation consists of the ability of transformed cells to proliferate even in conditions of restricted supply of growth factors and nutrients [56]. The unchecked proliferation of H-Ras V12 transformed cells was inhibited by exposure of these cells to the cell-penetrant anti-oxidant *N*-acetylcysteine. Thus, proliferation of these cells was directly dependent upon the concentration of superoxide. Moreover, overexpression of a dominant negative isoform of Rac1 (Rac1N17) inhibited both superoxide generation

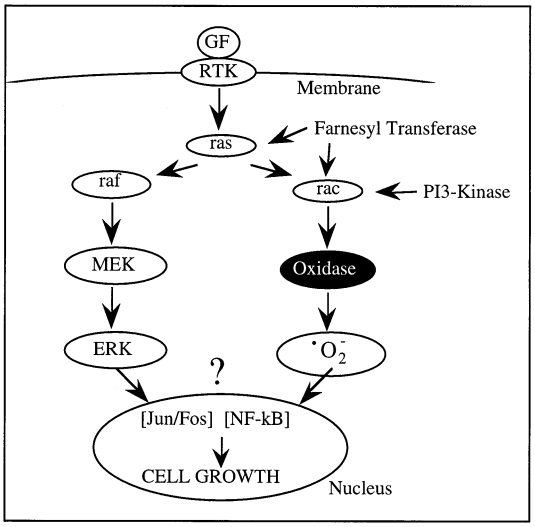

FIG. 4. Ras, superoxide and signal transduction. An alternative effector pathway for Ras is shown. The pathway includes Rac, an NADPH oxidase, and superoxide and shows specifically how Ras, Rac, and superoxide regulate mitogenesis and how cell growth remains essentially uncharacterized (question mark). Abbreviations: MEK, mitogen-activating protein kinase-kinase; ERK, MAP-kinase; NF-κB, nuclear factor-κB; RTK, receptor tyrosine kinase; and PI, phosphatidylinositol.

and unchecked proliferation. In contrast, NIH 3T3 fibroblasts transformed with a constitutively activated isoform of the serine and threonine kinase, Raf, neither produced detectable amounts of superoxide, nor were inhibited significantly in their proliferation by anti-oxidants.

Together, these data support the concept that NIH 3T3 cells transformed with H-Ras V12 produce superoxide constitutively, and that such production is required for their unchecked proliferation. Production of superoxide and proliferation in these chronically transformed cells were not dependent upon the Raf/MAP-K pathway, which, instead, seems to play a pivotal role in cells acutely activated by growth factors (Fig. 4) [15–17]. Cells chronically transformed with H-Ras V12 are probably undergoing a series of phenotypic changes, such as the overexpression of catalase [54], changes that might be required for their adaptation to altered redox conditions.

Several mechanisms could explain the contribution of superoxide to transformation by Ras. The mechanism accounting for the effect of oxidants on cell transformation has implicated the targeted damage of chromosomal DNA (or altered repair), leading to an enhanced rate of oncogenic mutations or, possibly, to the loss of tumor suppressor gene products [57, 58]. Through such a mechanism, the inhibition of tumor progression by anti-oxidants would be rather indirect and, to some extent, unpredictable. An additional mechanism can be proposed whereby superoxide and superoxide-derived ROS play a direct role in the signalling cascade that underlies transformation [54]. In this context, anti-oxidants predictably counteract pro-oncogenic signals. Several downstream effector molecules could be targeted by oxidants, either directly or indirectly, to induce transformation. ROS have been shown to activate nuclear factor-κB (NF-κB), a transcription factor whose activation has been linked to inhibition of apoptosis induced by cytokines like tumor necrosis factor-α (TNF-α) [59–62]. Hence, superoxide, and possibly other ROS, might contribute to unchecked proliferation through evasion of the immune surveillance by Ras-transformed cells. Although there is no evidence in mammalian cells, for proteins capable of "sensing" superoxide or H_2O_2 (as can be found in prokaryotes) [63], there is growing evidence supporting the concept that many signal-transducing proteins and transcription factors are highly sensitive to the redox state of the cells [54, 64–66]. The characterization of the molecular reactions responsible for such "sensitization" will require further work (Fig. 5).

RAS, RAC, AND THE CONTROL OF THE ACTIN CYTOSKELETON

Constitutively activated isoforms of Ras and Rac not only alter the mitogenic activity of cells, but they are also known for their strong impact on the organization of the actin cytoskeleton [73–75]. It is tempting to speculate that superoxide (and perhaps other ROS) generation, resulting from activation of Ras and/or Rac, also mediates the effects of small GTP-binding proteins on the actin cytoskeleton [71, 76]. Such effects could be mediated, either directly or indirectly, by interaction of ROS with the proteins of the actin cytoskeleton: (i) superoxide could affect the activity of molecules belonging to signal-transduction pathways regulating the actin cytoskeleton; consequently, interactions of regulatory proteins for actin with inositol phospholipids, calcium, and perhaps other signalling molecules known to be involved in actin regulation [77], might be altered; and (ii) alternatively, ROS could directly oxidize actin and/or binding proteins [71, 78]. Whatever the specific mechanism involved in the reorganization of the actin cytoskeleton by ROS might be, the net result of such interactions is likely to require the contribution of other, concurrently activated, signalling pathways [79, 80].

Interestingly, polyphosphoinositides such as phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] and phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P_3] have been proposed as regulatory molecules for actin-binding proteins and for the small GTP-binding proteins [81, 82]. Hence, superoxide (and other ROS) production resulting from the activation of Rac might function to amplify signals generated by the turnover of inositol phospholipids and targeted at the actin cytoskeleton [77, 79, 82]. Future research aiming at investigating these issues should further our understanding of the redox control of the superstructure of cells.

CONCLUSION

The role ascribed to superoxide and derived oxidants in biology is clearly expanding. By analogy with nitric oxide (NO), whose activity at low concentration is to transduce

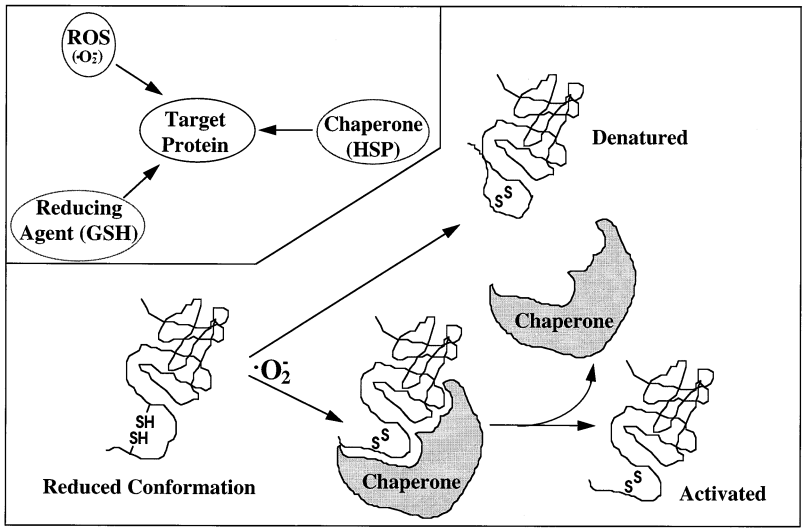

FIG. 5. Superoxide, chaperones and protein conformation. Cellular components are never exclusively exposed to oxidants. Instead, they are exposed to oxidants in a context where a large excess of reducing agents is also present, such as GSH, which functions as a true sulfhydryl buffer. Due to the very strong activity of the enzyme glutathione reductase, the oxidized conformation of glutathione (GSSG) is rapidly converted to GSH, such that the ratio of GSH to GSSG in most cells is maintained ≥ 500. Therefore, the oxidized conformation of targeted proteins is likely to be reduced rapidly by the abundant cellular anti-oxidants. Moreover, intracellular oxidants are promptly inactivated by dismutases, catalases, and cellular anti-oxidants. Hence, their oxidizing effects are expected to be limited, to a site directly surrounding their producing units. In addition, cells also contain large amounts of chaperone proteins, such as heat shock proteins (HSP). These protein-chaperones are known to protect their ligands against the damaging action of various stresses, including heat shock [67]. Thus, in the absence of chaperones, superoxide (and other ROS) might oxidize proteins to result in their denaturation, which would be followed rapidly by their degradation [68]. Instead, in the presence of chaperones, the titrated oxidation of targeted proteins like actin or other protein/enzymes might result in conformational changes that could contribute to their activation [69, 70]. Alternatively, the relative sensitivity of proteins to oxidants in a given system might result in the disruption of steady state for this system, resulting in substantial reorganization of the affected system [55, 71, 72].

signals within vessels and neurons [83], while high concentrations produce damage to cells and microorganisms [84], superoxide and probably other oxidants function as messengers at a low concentration, while larger amounts are required for cidal activity. In addition to cancer and infections, oxidants have been implicated in the genesis of many disease entities. For example, ROS production has been detected during tissue reperfusion after a period of ischemia, and has been shown to contribute to injury to the heart [85], the brain, [86], and the gastrointestinal tract [87], following acute myocardial infarction, stroke, or mesenteric ischemic insult. Through metal-catalyzed oxidation reactions, proteins can be denatured or cleaved [68]. In the pathogenesis of systemic sclerosis, oxidants have been shown to conspire with selected protein-bound metals to generate peptide fragments with antigenic properties that are able to break self-tolerance [88].

Perhaps more interesting will be the definition of the role of superoxide and other ROS as signal-transducing molecules involved in the remodeling of tissues, either spontaneously as a result of genetic alterations of the key regulatory proteins involved in free radical generating, degrading, and sensing pathways, or following specific injuries. In a transgenic mouse model, overexpression of H-Ras in the cardiac tissue results in a hypertrophic phenotype, with increased myofibrillar disarray, ventricular wall thickness, and juvenile mortality [89]. Overexpression of an intracellular enzyme that catabolizes superoxide, Cu,Zn-superoxide dismutase, protects tissues from ischemic injuries [90]. Though the production of oxidants by phagocytes has been traditionally implicated as the main source of superoxide and other ROS in injured tissues, the discovery of the widespread use by cells of oxidants as signalling molecules is likely to improve our understanding of the contribution of such molecules to the biology of normal and injured tissues. 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 triphosphatases of the Ras family.

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