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Reactive Oxygen Species in the Activation and Regulation of Intracellular Signaling Events

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I. Introduction

All aerobic cells are capable of producing reactive oxygen species (ROS) due to incomplete reduction of molecular oxygen to water under both physiological and pathological conditions (1). As unstable free radicals containing an unpaired electron in their outer orbital, ROS are extremely active in reaction with other cellular components, such as lipids, proteins, and DNA through electron capturing. Terminologically, ROS include both free radicals, such as superoxide anion ($^{\bullet}O_2^-$), hydroperoxyl radical ($HO_2^{\bullet}$), and hydroxyl radical ($^{\bullet}OH$), and non–free radicals, mainly H_2O_2. Although H_2O_2 by itself is not a free radical, in the presence of reduced transition metals (e.g., ferrous or cuprous ions), it can be transformed into the highly reactive $^{\bullet}OH$ and related oxidants through the Fenton reaction (2). Cellular sources of ROS include membrane-associated NADPH oxidase, cytosolic xanthine oxidase, peroxisomal oxidases, endoplasmic reticular oxidases, and mitochondria. Among these cellular sources, both NADPH oxidase and mitochondria are considered to be the major sources of ROS production in response to specific circumstances. In addition, a number of environmental hazards, such as chemical toxicants, carcinogenic metals, and fibrotic mineral dusts, can serve as either powerful exogenous sources of ROS or stimulate the production of ROS from cells (1). It has been realized

for decades that the production of ROS is a double-edged sword. On t one hand, ROS seem to be needed for signal transduction pathwa that regulate cell development, growth, and survival. On the ot hand, excessive generation of ROS, resulting from stress or exposure environmental hazards, can be responsible for or implicative in t development of numerous human diseases, such as degenerative disea inflammation, cancer, ischemia–reperfusion injury, and metabolic disord

This chapter will review current evidence that ROS activate intr cellular signaling pathways, which control the production of inflammato and proliferative mediators responsible for the initiation and progressio of diseases. Particular emphasis will be placed on the role of ROS in th induction of signals leading to the activation of phosphoinositide 3-kina (PI3K), nuclear factor κB (NF-κB), activator protein-1 (AP-1), and tumo suppressor p53.

II. Sources of ROS

A. Respiratory Burst and ROS

The term "respiratory burst" was first coined by Baldride and Gerard (3) in 1933 to describe an increase in oxygen consumption during ingestion of microorganisms by phagocytic cells. The key component of this respiratory burst system is the membrane-bound multisubunit enzyme complex termed the NADPH oxidase in both phagocytic cells and nonphagocytes (4,5). As a pivotal defense system against a range of infectious agents, activation of NADPH oxidase generates superoxide anion ($^{\bullet}O_2^-$), which in turn can generate other forms of ROS that are lethal for most microorganisms. NADPH oxidase is inactive in resting cells (6). Upon stimulation by a variety of soluble mediators and by particulate stimuli that interact with cell surface receptors, this oxidase is rapidly activated to produce $^{\bullet}O_2^-$ and other ROS, such as H_2O_2, $^{\bullet}OH$, and hypochlorous acid, through the one-electron reduction mechanism:

$$NADPH + H^+ + 2O_2 \xrightarrow{\text{NADP oxidase}} NADP^+ + 2H^+ + 2^{\bullet}O_2^-$$

Hydrogen peroxide is formed from the subsequent dismutation of superoxide:

$$2^{\bullet}O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Hydroxyl radical can be generated by the Haber–Weiss reaction:

$$^{\bullet}O_2^- + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + O_2$$

In the presence of myeloperoxidase, found in neutrophils, hypochlorous acid can be formed:

$$H_2O_2 + Cl^- \xrightarrow{\text{myeloperoxidase}} HOCl + H_2O$$

The core components of NADPH oxidase are five subunits: $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, $p22^{phox}$, and $gp91^{phox}$. In nonstimulated cells, three of these five subunits ($p40^{phox}$, $p47^{phox}$, and $p67^{phox}$) form a complex in the cytosol (6). The other two subunits, $p22^{phox}$ and $gp91^{phox}$, are dimerized as cytochrome b_{558} located in the membranes of secretory vesicles and specific granules. A small GTPase, Rac1/Rac2, may associate with cytochrome b_{558} to promote the interaction between $p67^{phox}$ and cytochrome b_{558} during the assembly and activation of NADPH oxidase (7,8).

The mechanism of NADPH oxidase assembly has been extensively investigated in the past few years. A major breakthrough is the discovery of the phox homology (PX) domain in the $p40^{phox}$ and $p47^{phox}$ subunits (Fig. 1) and the regulatory role of phosphoinositide (PI) on NADPH oxidase assembly. PI, produced from PI3K-activated lipid metabolism in response to extracellular stimuli, can recruit $p40^{phox}$ and $p47^{phox}$ through interactions with the PX domains (9,10). Then these cytosolic subunits can be translocated and assembled with the membrane-associated cytochrome b_{558} to form the active complex. The importance of PX domains in the assembly and activation of NADPH oxidase complex has been revealed by a

Figure 1 Alignment of phox homology (PX) domain sequence in $p40^{phox}$ and $p47^{phox}$.

mutation experiment. Mutation in the PX domain basic motif I of $p47^{phox}$ blocks the interaction with PtdIns(3,4)P_2 and the generation of superoxides.

Molecular modeling studies by analysis of the solution structure demonstrated that the SH3 domain in the C-terminal of $p47^{phox}$ is critical in determining intramolecular interactions. This SH3 domain can bind to the PxxP motif within the helical lobe of the $p47^{phox}$ PX domain and can induce a conformational change in the PX domain (11). Since the PxxP motif, the membrane interaction loop, and the PI-binding PX domain are structurally adjacent, the interaction between C-terminal SH3 and PxxP may sterically cause $p47^{phox}$ to be in an auto-inhibited state. The binding of PtdIns(3,4)P_2 releases the SH3 domain from the PX domain and promotes the association of $p47^{phox}$ with the PxxP motifs of other NADPH subunits.

An additional important mechanism for the regulation of NADPH oxidase assembly and activation is phosphorylation (12). It has been noted for several years that the cytosolic component $p47^{phox}$ becomes heavily phosphorylated in the cellular response to a wide variety of stimuli. This phosphorylation of $p47^{phox}$ is thought to disrupt the PX-SH3 domain–mediated intramolecular associations to expose previously masked domains, which allows direct interaction of $p47^{phox}$ with the $p22^{phox}$ subunit in the cytochrome b_{558} complex (6). The phosphorylation of $p47^{phox}$ appears to be required for the entire cytosolic complex to migrate to the membrane where cytochrome b_{558} is located. A number of kinases have been shown to be able to phosphorylate various serine residues in the C terminus of $p47^{phox}$. These kinases include protein kinase C (13,14), mitogen-activated protein kinases (MAPK), extracellular signal-related kinase (ERK) (15) and p38 (16), protein kinase A (PKA) (12), and p21-activated kinases (PAKs) (17). Phosphorylation of serine 379 (Ser379) has been shown necessary for both the translocation of $p47^{phox}$ and the activation of NADPH oxidase (18). Recent studies have found that phosphorylation of Ser303, Ser304, Ser359, and Ser370 is also involved in the process of assembly and activation of NADPH oxidase (14,19,20). Mutation of these serines, either individually or in combination, greatly decreases oxidase activity. Surprisingly, mutation of MAPK target pair Ser345/Ser348 does not affect the activity of NADPH oxidase (21). In addition to $p47^{phox}$, the $p40^{phox}$ and $p67^{phox}$ subunits have also been shown to be phosphorylated in cells treated with functional microsomal lipoprotein (fMLP) and phorbol, respectively (22,23). However, the functional consequences of $p40^{phox}$ and $p67^{phox}$ phosphorylation have not been well established.

The ROS generated from NADPH oxidase activation have been associated with intracellular signal transduction from the activation of transcription factors to the regulation of cell growth. Furthermore, the PX domains have been identified in a number of other cellular signaling proteins

Table 1 PX Domain–Containing Proteins

Protein	Function
$p40^{phox}$	NADPH oxidase
$p47^{phox}$	NADPH oxidase
PI3K C2-γ	PI3K signal transduction
PLD1	Exocytosis
CISK	Akt signaling, cell survival
RGS	G-protein signaling
Vam7	Protein sorting and trafficking
SNX1	Protein sorting and trafficking
SNX2	Protein sorting and trafficking
SNX3	Protein sorting and trafficking
SNX4	Protein sorting and trafficking
SNX6	Protein sorting and trafficking
SNX7	Protein sorting and trafficking
SNX15	Protein sorting and trafficking
SNX16	Protein sorting and trafficking

in addition to the NADPH oxidase subunits, $p40^{phox}$ and $p47^{phox}$ (Table 1) (24). Therefore, the respiratory burst due to the activation of NADPH oxidase may affect pathways controlling many cellular responses.

B. Mitochondria: The Major Cellular Site for ROS Generation

ROS are commonly generated as byproducts of the mitochondrial electron transfer reaction for the production of ATP (25). Evidence suggests that the formation of $^{\bullet}O_2^-$ by mitochondria is through both respiratory chain at the inner mitochondrial membrane and the outer mitochondrial membrane-associated monoamine oxidases (26). The respiratory chain receives electrons from either NADH or flavoprotein-linked dehydrogenases and ultimately reduces oxygen to water by four electron-transporting complexes (I–IV) and one H^+-translocating ATP synthetic complex (complex V). However, it is believed that about 2–4% of the total oxygen consumed during electron transport is reduced not to water by cytochrome *c* oxidase but rather to $^{\bullet}O_2^-$ due to the "leakage" of unpaired electrons to molecular oxygen during the proton-motive quinone cycle (26). The sites of $^{\bullet}O_2^-$ formation in respiratory chain include complex I (NADH/ubiquinone oxidoreductase) and complex III (ubiquinol/cytochrome *c* oxidoreductase). This proposal is based on the fact that the flavin mononucleotide of complex I and ubiquinone in complex III can exist in a semiquinone (SQ) anion form,

which contains an unpaired electron that can be donated to molecular oxygen to form $^{\bullet}O_2^-$.

Mammalian complex I is a multiple subunit complex comprising 34 proteins encoded by the nuclear DNA and 7 proteins encoded by mitochondrial DNA (27). The catalytic core of complex I includes PSST, TYKY, 49-kDa, ND1, and ND5 subunits. The mechanism of $^{\bullet}O_2^-$ production by complex I is possibly through the generation of SQ within complex I. As molecules with an unpaired electron, semiquinones are the likely electron donors for transforming O_2 to $^{\bullet}O_2^-$. Biochemical evidence indicates that there are three distinct species of SQ forms, i.e., SQNf (fast-relaxing component), SQNs (slow-relaxing component), and SQNx (very-slow-relaxing component), formed in complex I (28,29). The generation of $^{\bullet}O_2^-$ by complex III is also mediated by the formation of SQ catalyzed by several key subunits in this complex, such as cytochrome *b*, the Rieske iron–sulphur protein, and cytochrome c_1 (30).

Under the physiological condition, the $^{\bullet}O_2^-$ generated by the respiratory chain in mitochondria is scavenged by SOD_2 (MnSOD) in the mitochondrial matrix to form H_2O_2. Since mitochondria do not contain catalase, glutathione (GSH) peroxidase is the only enzyme that detoxifies H_2O_2 to water in a reaction that converts reduced GSH to oxidized GSSG. However, under the nonphysiological conditions, such as exposure to environmental insults, ischemia–reperfusion, or hyperoxia, excessive $^{\bullet}O_2^-$ can become highly damaging to a number of mitochondrial and cytosolic proteins, especially to those containing 4Fe-4S iron–sulfur center(s). It has been demonstrated that $^{\bullet}O_2^-$ itself is capable of inactivating mitochondrial NADH dehydrogenase, NADH oxidase, aconitase, and ATPase (25). Furthermore, the reaction of $^{\bullet}O_2^-$ with the 4Fe-4S center of these mitochondrial proteins results in the release of ferrous ion, which facilitates the generation of $^{\bullet}OH$ from H_2O_2 through the Fenton reaction. $^{\bullet}OH$ is an active free radical that can damage lipids, proteins, nuclear DNA, and/or mitochondrial DNA (mtDNA). Oxidative damage of mitochondrial proteins or mtDNA will result in higher rates of $^{\bullet}O_2^-$ production.

C. Exogenous Sources of ROS or ROS Amplifiers

ROS can be found in atmospheric air, most likely due to the ultraviolet-mediated photolysis of ozone (31). It is estimated that 1 L of normal atmospheric air on a sunny day contains more than one billion $^{\bullet}OH$ (32). A number of environmental factors, such as ultraviolet light, ionizing radiation, ozone, transition metals, combustion smoke, pesticides, certain industrial solvents, asbestos, and silica, can serve as exogenous sources of

ROS. After interaction with tissues or cells, these environmental ROS can further stimulate the ROS generation endogenously.

One of the most established environmental factors that not only acts as an exogenous source of ROS but also as an amplifier for intracellular ROS generation is crystalline silica particles (33). It is assumed that ROS are important mediators for the silica-induced fibrotic or carcinogenic effect in human lung. First, silica can spontaneously catalyze the formation of ROS in aqueous solutions (34). Second, interaction of silica particles with cell membranes induces lipoprotein oxidation and the subsequent NAD(P)H oxidase–mediated increase in intracellular ROS (35–37). Third, cellular uptake of silica particles promotes the oxidative burst (38).

Several free radicals or peroxides derived from silica particles or the interaction of silica with lung cells have been reported, such as $^{\bullet}OH$, $^{\bullet}O_2^-$, nitric oxide $^{\bullet}NO$, and H_2O_2 (39,40). A number of studies suggested that the unique surface characteristics of silica particles were critical for the generation of ROS in biological systems (41). The basic structural unit of most forms of silica is the silicon atoms surrounded by an approximately tetrahedral array of oxygen atoms (42). On the surface of the freshly fractured silica particles, the silicon–oxygen bonds (Si-O) are ruptured, which leaves an unpaired electron on both the Si and O atoms. In addition, the distribution and abundance of silanol (SiOH) groups, due to the hydration of Si-O bonds in aqueous solutions, have been shown to be important in the potency of ROS generation (43). ROS generation by silica was decreased when the silica particles were coated with polymers or aluminum lactate to modify or neutralize the silanol groups (44–46).

It has also been implicated that the existence of a trace amount of iron on the silica surface will potentiate the produce of ROS through the Fenton reaction (47). Chelation of the surface-associated trace iron decreased $^{\bullet}OH$ generation (43). Nevertheless, it is still debatable whether the presence of iron on the surface of silica particles is critical for the silica-induced tissue damage. Donaldson et al. (44) compared the amount of iron, ROS generation, and toxicity among several different quartz samples and indicated a reduced toxicity of quartz that contains more iron and generates more ROS. Similarly, a report by Porter et al. (48) demonstrated that although freshly fractured silica induced more ROS generation, both aged and fresh silica caused a similar degree of lung damage in rats.

It has been assumed that ROS directly generated from silica particles in aqueous solution could somehow contribute to the intracellular formation of ROS. However, recent studies by Deshpande et al. (49) do not support this hypothesis. Addition of catalase and/or SOD into silica-treated culture medium significantly diminished H_2O_2 in the medium. Following the centrifugation to remove the silica particles from the

medium, the supernatant was added to an epithelial cell culture. Intriguingly, treatment of the cells with this conditional medium unexpectedly increased intracellular ROS generation, suggesting that ROS formed from silica–medium interaction might be not accountable for the induction of intracellular ROS. It is unknown which fluid-phase factors were really responsible for this activation.

III. ROS and Signal Transduction

A. Relationship Between ROS and PI3K

The PI3K pathway has been shown to be capable of regulating a variety of cellular processes, including cell proliferation, growth, differentiation, apoptosis, membrane trafficking, and cytoskeletal rearrangement (50). There are three classes of PI3K, of which the class I PI3K has been studied most. PI3Ks are heterodimers composed of a p85 regulatory subunit and a p110 catalytic subunit. The p85 regulatory subunit, a phosphoprotein substrate of several cytoplasmic and receptor tyrosine kinases, can directly associate with many active tyrosine kinases or adaptor proteins through the interaction of its two Src homology 2 (SH2) domains with phosphotyrosine residues in the consensus YXXM motif on these kinases or adaptor proteins. This association may subsequently relieve an inhibitory effect of p85 on p110 kinase activity and bring the p110 catalytic subunit to its lipid substrates in the cell membrane. The active form of PI3K can phosphorylate the D-3 position of the inositol ring of phosphoinositides to form second messengers in cells. The products formed from PI3K activation include phosphatidylinositol (PtdIns)(3)P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3, which can recruit signaling proteins that contain PtdIns-binding domains such as the PX, Pleckstrin homology (PH), ENTH, and FYVE (51).

The currently accepted vision regarding the relationship between ROS and PI3K is that activation of PI3K causes ROS generation. This assumption is largely based on the fact that PtdIns from PI3K-catalyzed reactions facilitate the assembly of the NADPH oxidase complex through the recruitment of PX-containing $p40^{phox}$ and $p47^{phox}$ subunits. Indeed, a number of reports demonstrate that pharmacological inhibition of PI3K decreases $^{\bullet}O_2^-$ production or the respiratory burst in neutrophils or other types of cells (52–56). The direct evidence supporting the regulatory action of PI3K on respiratory burst activity is from the studies of knockout mice. The production of $^{\bullet}O_2^-$ is blocked in the chemoattractant-treated neutrophils isolated from PI3Kγ-deficient mice (57–59). However, cells from these PI3Kγ-deficient mice can still produce $^{\bullet}O_2^-$ in response to

lipopolysaccharides, suggesting that the involvement of PI3K in the respiratory burst is limited to selected pathways (59).

Most studies suggest that PI3K appears to be an upstream kinase of the respiratory burst, whereas several reports indicate the reverse is true, i.e., ROS activate PI3K. The evidence indicating that ROS activate PI3K is from the recent work of Wang et al. (60) who demonstrated that H_2O_2 induced activation of PI3K in HeLa cells or NIH3T3 cells. Inhibitors for PI3K have been shown to be able to inhibit the activation of Akt in these cells following stimulation with H_2O_2. Indeed, in the course of their study, Wang et al. (60) showed that in both NIH3T3 cells and HeLa cells the Akt activation in response to H_2O_2 was blocked not only by wortmannin and LY294002, two pharmacological PI3K inhibitors, but also by a mutant form of the p85 subunit of PI3K that does not bind to the p110 catalytic subunit to direct its activation.

The activation of PI3K by ROS has also been demonstrated in skeletal myotubes (61), neurons (62), and human embryonic kidney 293 cells (63). In addition, a recent study by Kang et al. (64) reported that in vitro exposure of macrophages to silica could activate PI3K and that this activation was sensitive to inhibition by antioxidants. They concluded that ROS-induced activation of PI3K plays an important role in activation of signaling pathways involved in silica-induced inflammation. Since most of the studies suggesting that ROS activate PI3K have used exogenous H_2O_2 as a stimulant, the questions remaining are whether endogenous ROS activate PI3K and how ROS activate PI3K. It has been well established that PI3K activation is dependent on certain types of tyrosine kinases. Thus, it is possible that ROS derived from the respiratory burst or mitochondria may regulate PI3K by affecting tyrosine kinases or tyrosine kinase phosphatases.

B. Involvement of ROS in NF-κB Activation

NF-κB is a transcription factor governing the expression of a wide range of early response genes involved in cell-to-cell communication, cell movement, cell growth control, and inflammatory responses (65,66). At least five mammalian NF-κB gene family members have been cloned currently. These members include *nfkb1*, *nfkb2*, *relA*, *relB*, and *c-rel*. The most abundant and well-characterized activated form of NF-κB is a heterodimer composed of a RelA and a p50, a partially degraded product encoded by *nfkb1* gene. In resting cells, the majority of NF-κB complexes is retained in cytoplasm by binding with its endogenous inhibitor proteins including IκBα, IκBβ, IκBγ, IκBε, Bcl-3, and the precursor Rel proteins p100 and p105. All NF-κB

inhibitors contain multiple ankyrin repeats, which are responsible for the interaction with Rel homology domains (RHDs) of NF-κB.

A wide range of extracellular stimuli, such as inflammatory mediators, mitogens, toxicants, and microorganism products, induce NF-κB through the activation of an upstream kinase cascade. The key component of this signal transduction pathway is IκB kinase complex composed of two serine/thronine kinase subunits, IKKα and IKKβ, and a structural subunit, IKKγ/NEMO (65,66). Activation of IKK results in the phosphorylation of IκBα protein, a prerequired step for the subsequent ubiquitination and proteasomal degradation of this inhibitory protein.

ROS have long been known to activate NF-κB under many circumstances. The first evidence indicating NF-κB activation by ROS is from the study by Schreck et al. (67) who demonstrated that H_2O_2 could rapidly activate NF-κB. Using *N*-acetyl-L-cysteine, a well-characterized antioxidant, the NF-κB activation induced by H_2O_2, as well as several other inducers, was substantially inhibited. In a recent study, Jaspers et al. (68) explored the mechanism of ROS-induced NF-κB activation. They found that H_2O_2 stimulated IKK kinase activity in human bronchial epithelial cells, which correlated with the phosphorylation and ubiquitination of IκBα protein. In biochemical experiments with IKK mutants, the activation of IKK and NF-κB by H_2O_2 was decreased (69). Furthermore, H_2O_2 enhanced phosphorylation of serine 180 of IKKα and serine 181 of IKKβ in the activation loop.

Taking a molecular genetic approach, Fan et al. (70) obtained evidence that NADPH oxidase was essential for signal-induced NF-κB activation. They found that NF-κB activation by tumor necrosis factor-α (TNF-α) was significantly attenuated in neutrophils from mice with NADPH subunit $p47^{phox}$ gene knockout. In these $p47^{phox-/-}$ mice, the TNF-α-induced degradation of IκBα in lung tissue was dramatically decreased, suggesting impairment of upstream kinase activation. Similarly, disruption of NADPH oxidase assembly by expression of a dominant negative Rac1 in macrophages diminished the activation of NF-κB by lipopolysaccharides (71).

Stimulation of the respiratory burst produces not only ROS but also a number of other metabolic products, such as neopterin and its derivatives. Several reports suggest that these metabolic products are capable of activating NF-κB. In vascular smooth muscle cells, neopterin-induced inducible nitric oxide synthase (iNOS) gene expression was thought to be dependent on the activation of NF-κB (72). In human macrophages, neopterin amplified HIV-1 replication due to the activation of NF-κB, which recognizes a central enhancer element of the HIV LTR promoter (73). Neopterin also induced ICAM-1 expression in alveolar epithelial cells in a NF-κB-dependent manner (74).

The activation of NF-κB by exogenous sources of oxidants has been demonstrated in variety of cellular systems in response to silica (75–79), asbestos (80), glass fiber (81), chromium(VI) (82,83), vanadium(V) (84), and arsenite (85,86). Based on the fact that catalase or $^{\bullet}OH$ scavengers blocked silica-induced NF-κB activation, studies by Chen et al. (76) and Kang et al. (79) suggested that ROS are important mediators during this process. The underlying mechanisms of silica-induced NF-κB activation were further delineated by Kang et al. (77) who demonstrated that silica was capable of inducing tyrosine phosphorylation of IκBα protein in mouse macrophage cells. The phosphorylation of tyrosine residue (Tyr42) at the N terminus of IκBα was previously shown as an alternative mechanism of NF-κB activation in the cellular response to oxidative stress (87). In T cells, Tyr42-phosphorylated IκBα could be sequestered from the NF-κB complex through the association with the p85 subunit of PI3K subunit (88). Similarly, a recent study by Kang et al. (64) indicates that silica induces association of IκBα with p85 subunit of PI3K in mouse macrophage cells.

One might speculate, based on the above description, that ROS generation or oxidative stress is an essential step for the activation of NF-κB induced by a variety of signals. However, this assumption has been greatly challenged recently. The main argument for this challenge is the observation that oxidation of NF-κB proteins attenuates the DNA binding activity of this transcription factor (89–92). Furthermore, studies by Korn et al. (93) demonstrated that oxidation of the conserved Cys 179 in the kinase domain of IKKβ inactivated the kinase activity. It is possible that the conflicting effects of ROS occur on different levels of NF-κB activation cascade. While ROS are inhibitive for the DNA binding activity of NF-κB and the kinase activation of IKK, ROS may activate or potentiate the activation of other kinases that integrate into the NF-κB signaling. Nevertheless, despite a large number of reports suggesting the involvement of ROS in the activation of NF-κB, it is still elusive as to which kinase or molecule in this signaling pathway serves as an ROS sensor for the activation of NF-κB.

C. ROS and Activator Protein-1

AP-1 was originally identified as an oncological basic domain/lucine zipper (bZIP) transcription factor based on the fact that it contains c-Jun or c-Fos oncoprotein and binds to the enhancers of SV40 in response to the tumor promoter tetradecanoyl phorbol acetate (TPA). It is well documented that AP-1 is composed of homo- or heterodimers of the protein products of individual members within the Jun (c-Jun, c-JunB, and c-JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) immediate–early response gene families.

Both c-Jun and c-Fos proteins contain several transactivation domains and an interacting region with the TATA box–binding protein. The c-Fos and Fra-1 proteins contain an additional carboxy terminal transrepression domain (94). As a sequence-specific transcription factor, AP-1 can bind to the TPA response elements (TREs) in the promoter or 5′ flanking regions of a number of target genes. The target genes regulated by AP-1 include those encoding c-Jun, Egr1, Egr2, collagenase, stromelysin, certain cytokines, cyclin D, transforming growth factor-β, and a number of other genes involved in both inflammation and carcinogenesis.

It has been more than a decade since the first demonstration indicating the redox regulation of AP-1 DNA binding activity (95). Oxidation of the conserved cysteines (Cys154 in Fos and Cys272 in Jun) in the DNA-binding domain decreases the DNA binding efficiency and the transforming activity of AP-1. In contrast, treatment of the cells with reducing agents, such as *N*-acetyl-L-cysteine, and pyrrolidinedithiocarbamate (PDTC), enhances the DNA binding and transcriptional activity of AP-1 (96). Paradoxically, a number of studies have demonstrated that certain oxidants, such as asbestos, silica, chromium, ultraviolet light, and H_2O_2, activate AP-1 in a variety of cellular systems or animal models (97–101). For instance, in a mouse JB6 epidermal cell line, freshly fractured silica, which is more potent than aged silica in inducing ROS generation, caused an eightfold increase in AP-1 activity (98,102). Similarly, silica induces AP-1 activity and the mRNA accumulation of AP-1 target genes in a nontumorigenic murine alveolar type II epithelial cell line, and this stimulation can be inhibited by antioxidants (97). This raises an interesting question of why AP-1 is activated by oxidants that under certain conditions can actually inhibit the transcriptional activity of AP-1.

It has been noted for many years that a number of extracellular stimuli activate AP-1 through the posttranslational mechanisms without the requirement of new protein synthesis (103). Thus, certain redox-sensitive intracellular signal transduction pathways may be responsible for the activation of AP-1 by ROS. Activation of these signaling pathways changes the phosphorylation patterns of the AP-1 subunits. Currently, this phosphorylation mechanism is being investigated in great detail for the regulation of Elk-1, Jun, ATF-2, and MEF2 function in the AP-1 complex. Three key MAPK pathways—ERK, JNK, and p38—have been identified in mammalian cells. Functional MAPK circuits are three-tiered kinase modules. The Raf-MEK-ERK module is employed ubiquitously in the cellular response to growth or differentiation signals relayed by receptor tyrosine kinases or G-protein-coupled receptors. Signaling through JNK mediates oxidation, DNA damage, and a variety of other types of stress responses, whereas p38 is mainly involved in certain inflammatory reactions

induced by cytokines or apoptotic factors. Despite the fact that each MAPK has its preferential stimuli, all three MAPKs are sensitive to ROS.

ROS Regulation of JNK

In response to a variety of environmental stress signals, eukaryotic cells use a JNK cascade leading to the activation of a number of cellular events (104). JNK can phosphorylate the conserved Ser63 and Ser73 residues at the NH_2 terminal activation domain of c-Jun, which associates with c-Fos protein to form the AP-1 transcription factor. Three genes, *jnk*1, *jnk*2 and *jnk*3, have been identified to encode JNK (104). Several upstream kinases, such as ASK1, MEKK, MLK, TAK1, and TPL-1, activate JNK by MKK7 or MKK4, which phosphorylates Thr183 and Tyr185 of JNK. The activation of JNK has been linked to the intracellular signal transduction and gene expression involved in the regulation of cell proliferation, apoptosis, and carcinogenic transformation.

Activation of JNK by ROS has been observed in the cells exposed to UV-C and by overexpression of the $p21^{ras}$ oncogene (105). In primary bovine articular chondrocytes, the activation of JNK by cytokines interleukin-1 (IL-1) and TNF-α was thought to be through NADPH oxidase–dependent ROS generation (106). Evidence supporting this conclusion includes the following: (a) cytokine-induced JNK activation could be attenuated by the antioxidant *N*-acetyl-L-cysteine; (b) exogenous H_2O_2 mimicked the effect of cytokines on the activation of JNK in this cell type; (c) inhibition of flavonoid-containing enzymes, such as NADPH oxidase, partially blocked cytokine-induced JNK activation. These observations are consistent with the earlier reports indicating that Rac1, a small GTPase protein involved in the assembly of NADPH oxidase, was an upstream regulator of JNK (107–109).

In nonphagocytic cells, a recent study by Gu et al. (110) demonstrated that the TNF-α-induced JNK activation was also through the activation of NADPH oxidase. In living cells, they found that $p47^{phox}$, a cytosolic NADPH oxidase subunit, was concentrated on the cortical cytoskeleton. TNF-α treatment caused membrane translocation of $p47^{phox}$. Intriguingly, disruption of the cytoskeleton decreased both oxidant production and JNK activation by TNF-α. Expression of a mutant $p47^{phox}$ diminished JNK activation by TNF-α. A logical conclusion from the above finding is that activation of JNK by TNF-α must be preceded, at least in the TNF-α-stimulated nonphagocytic cells, by the assembly and activation of NADPH oxidase.

The mechanisms whereby ROS activates JNK are being elucidated. JNK itself does not appear to be the direct target of ROS because ROS was unable to activate immobilized JNK (106). Therefore, a redox-sensitive

target must exist in the activation pathway for JNK. The first target of ROS in the activation of the JNK pathway is the apoptosis signal-regulating kinase 1 (Ask1) (111). Ask1 is a member of the MAPKKK family that activates JNK via activation of MKK4/SEK1. In resting cells, Ask1 is inactivated by association with its endogenous inhibitor, thioredoxin (Trx), that binds to the amino terminal noncatalytic domain of Ask1 and blocks activation of Ask1 by TNF-α (112). Oxidative stress, such as H_2O_2 treatment, will oxidize Trx at several cysteine residues and dissociate Trx from Ask1 and thereby activate Ask1. Furthermore, a new model of Ask1 activation by ROS has been proposed by Tobiume et al. (113) who suggested that oxidative stress could induce the phosphorylation of Thr845 in the activation loop of Ask1. This phosphorylation of Thr845 appears to be required for the full activation of Ask1 by creating a conformational change of the Ask1 oligomer.

The second target of ROS in the JNK activation cascade is possibly the glutathione-*S*-transferase pi (GSTp) (114). In normally growing nonstressed cells, JNK is inhibited by direct binding with the monomer GSTp (114). ROS elicited by UV or H_2O_2 can switch the GSTp from a monomeric to a dimeric/multimeric form. Because of disulfide bond–induced steric constraints, dimerized GSTp cannot associate with JNK. Since the overexpression of GSTp has been associated with transformation to malignancy (115), it is possible that GSTp-mediated JNK inhibition may facilitate the tumor cells to escape from apoptosis.

Finally, ROS may also inactivate the dual-specificity threonine-tyrosine phosphatase M3/6 (also named hVH-5) that dephosphorylates and inactivates JNK (116). The only evidence that directly supports this is from the study by Chen et al. (117). In Jurkat, HEK293, LNCaP, and Tsu-Pr1 prostate cancer cells, H_2O_2 activated JNK by targeting phosphatase M3/6. However, it is interesting to note that H_2O_2 significantly decreased the expression levels of phosphatase M3/6, but not the levels of other phosphatases (PP2A and PP4). In contrast, UV-C irradiation did not cause the down-regulation of phosphatase M3/6. It is unknown whether this down-regulation of phosphatase M3/6 is due to oxidation-facilitated degradation or inhibition of phosphatase M3/6 expression.

ROS Regulation of ERK

The ERK pathway is generally linked to the regulation of cell proliferation or survival rather than stress regulated by JNK or p38. However, during oxidative stress induced by excessive accumulation of ROS, the activation of ERK, JNK, and p38 can occur in a coordinate fashion (118). This observation was supported by the studies of silica-induced activation of three MAPKs (97,98). Although silica induced a dramatic activation of JNK

in rat nontransformed alveolar type II epithelial cell line, the activity of ERK was not diminished (97). Similarly, in mouse JB6 epidermal cells, silica induced phosphorylation of ERK and p38 (98). The AP-1 activity induced by silica in this cell type could be attenuated by both ERK and p38 inhibitors (98). The ROS-dependent activation of ERK has also been seen in the cellular responses to asbestos (119), short-wave UV light (120), arsenite (121), and H_2O_2 (119). How ROS activate ERK is unclear, but two possible mechanisms may be involved. First, ROS may lead to the ERK activation through several growth factor receptors, including epidermal growth factor receptor (122) and platelet-derived growth factor receptor (123). The evidence supporting this notion is from the experiments in which expression of inactive mutant forms of various growth factor receptors reduces ROS-induced ERK activation (124). It was believed that oxidants might modify the cysteine residues on the receptors to mimic the effects of ligand–receptor interaction (125). Second, ROS may inactivate the membrane-associated protein phosphatases necessary for dephosphorylation of growth factor–signaling mediators (126). Inactivation of phosphatases will lead to a sustained phosphorylation and activation of growth factor signaling.

ROS Regulation of p38

MAPK p38 consists of at least five subfamily members: α, β_1, β_2, γ, and δ. The activation of p38 has been observed in coordination with JNK activation in a number of types of cells treated with oxidants including silica (98), asbestos (127), Cr(VI) (100), H_2O_2 (128), and UV light (129). Thus, a common upstream signaling pathway may be involved in ROS-induced activation of both p38 and JNK. It has been generally believed that Ask1 serves as a sensor for ROS. As mentioned earlier, Ask1 is a kinase, acting upstream of MKK4, and MKK3/6, which leads to activation of JNK and p38, respectively. Using embryonic fibroblasts derived from Ask1 gene knockout mice, Tobiume et al. (130) demonstrated that the H_2O_2-induced sustained activation of JNK and p38 is lost in Ask1(-/-) cells. In B cells, the activation of p38 by ROS was thought to be through the phospholipase Cγ2–dependent activation of Syk, a nonreceptor tyrosine kinase widely expressed in hematopoietic cells (131). In cardiomyocytes, mitochondria ROS release is considered to be critical in the activation of p38 during hypoxia (132). Inhibition of either the mitochondrial respiratory chain or the mitochondrial membrane anion channel prevents the activation of p38 by hypoxia. The ROS-induced activation of p38 may be responsible for cell cycle arrest at G2/M phase (133) due to the phosphorylation and inhibition of Cdc25B and Cdc25C, two important cell cycle regulatory proteins for G2/M phase transition (134). In addition, activation of p38 might also

contribute to the ROS-induced cell apoptosis, possibly through the phosphorylation of p53 (135).

It is interesting to note that while p38 can be activated by ROS, p38 can amplify the generation of ROS through the assembly of NADPH oxidase (136). In bovine neutrophils, $^{\bullet}O_2^-$ production was induced by stimulation with serum-opsonized zymosan and attenuated by the p38 inhibitor SB203580, which blocked the translocation of $p47^{phox}$ and Rac to the plasma membrane (136). This notion suggests an important consequence of ROS-induced p38 activation that further increases the level of ROS generation or oxidative stress. This positive-feedback loop may be critical in the cellular commitment to apoptosis in response to ROS.

IV. Is p53 Regulated by or a Regulator of ROS?

Wild-type p53, a transcription factor sensitive to a variety of genotoxic stresses, governs the expression of genes involved in tumor suppression through cell cycle arrest and apoptosis (137). The genes whose expression is regulated by p53 include G1 arresting gene, $p21^{waf1}$ (138), G2/M phase arrest genes GADD [growth arrest- and DNA damage-induced gene] 45α (139) and 14-3-3σ (140), and the proapoptotic genes encoding Bax (141), Fas (142), DR5 (143), Noxa (144), p53-upregulated modulator of apoptosis (PUMA) (145,146), p53AIP1 (147), and p53-induced protein with death domain (PIDD) (148). Activation of p53 following genotoxic stress or environmental insults occurs largely through posttranslational modification, such as phosphorylation (149), acetylation (150), ubiquitination (151), and SUMO [small ubiquitin-related modifier]rization (152). These modifications affect the stability and DNA binding activity of p53 proteins.

Accumulating evidence suggests that ROS regulation of p53 is complicated (153). One of such means is the activation of upstream kinases, such as DNA damage–dependent protein kinase (DNA-PK), ataxia-telangiectasia mutated (ATM) kinase, ATM-related kinase (ATR), and possibly checkpoint kinase1/2 (Chk1/2). These kinases can phosphorylate a variety of serine residues on p53 protein. It has been well documented that ROS generated either from genotoxic stress or nongenotoxic damage can cause oxidative damage of DNA molecules, which in turn activates DNA-PK or ATM. A recent study by Xie et al. (154) demonstrated that H_2O_2-induced phosphorylation of p53 at multiple serine residues was blocked in ATM-deficient cells. Regulation of p53 phosphorylation by ATM may be partially through the activation of polo-like kinase-3 (Plk-3) (154) and Chk2 (155). Both Plk-3 and Chk2 are capable of phosphorylating Ser20 at the N terminus of p53 in an ATM-dependent manner following

ionizing radiation or UV-C treatment (154,156). Phosphorylation of p53 on the N-terminal serines interferes with the association of p53 with its inhibitor, Mdm2, a ubiquitin ligase targeting p53 for proteasomal degradation (157).

The stability of p53 protein may be directly regulated by the intracellular redox status. Evidence supporting this comes from the observation that NAD(P)H quinone oxidoreductase-1 (NQO1) inhibits the degradation of p53, especially under oxidative stress conditions (158). NQO1 is a ubiquitously expressed two-electron reductase with NAD(P)H as an electron donor. Inhibition of NQO1 by dicoumarol facilitates the degradation of p53 by proteasome. Overexpression of NQO1, on the other hand, stabilizes p53 protein. However, NQO1 does not affect the stability of β-tubulin, IκB, or poly(ADP-ribose) polymerase, suggesting a certain level of specificity of NQO1 regulation on p53. These findings are consistent with an earlier report indicating that overexpression of WOX1, another oxidoreductase, can increase the p53 protein level in L929 cells (159).

An additional measure of ROS regulation of p53 is the direct oxidative modification of critical cysteine residues in the DNA-binding domain of the protein, thus regulating its DNA binding activity (153). One of the most notable features of the DNA-binding domain of p53 is the large loop-helix structures bridged together by the tetrahedral coordination of a divalent zinc atom on Cys176, His179, Cys238, and Cys242 (160). Thus, oxidation of the thiol group of these cysteine residues disrupts the zinc bridge and causes conformational changes of the DNA-binding domain of p53. Indeed, evidence from the use of thiol oxidants, such as diamide, suggests that oxidation of these cysteine residues abrogates DNA binding of p53 (161). However, there are several conserved cysteine residues other than those involved in the binding of zinc in the DNA-binding domain of p53. These cysteine residues may be more accessible for redox modification. A recent study by Buzek et al. (162) suggests that oxidation of Cys277 in the DNA-binding domain of p53 decreases the affinity of p53 toward the p53-responsive element of *gadd45α* gene. In contrast, Cys277 oxidation did not affect the binding of p53 to the p53-responsive element of the $p21^{waf1}$ gene. Thus, oxidation may not only affect the DNA binding activity of p53 but may also allow p53 to discriminate among individual responsive elements in various target genes and shift the spectrum of gene expression regulated by p53.

It is important to note the existence of a positive-feedback mechanism of ROS-induced p53 activation. In certain types of cells, activation of p53 increases the levels of intracellular ROS. Several genes involved in the redox regulation have been shown to be transcriptionally regulated by p53. Using a SAGE assay, Polyak et al. (163) investigated the effect of overexpression

of wild-type p53 protein. In the colorectal carcinoma cell line DLD-1, overexpression of p53 increased the expression of quinone oxidoreductase (pig3), proline oxidase homology (pig6), and glutathione transferase (pig12). In addition, p53 has also been shown to induce the expression of glutathione peroxidase (GPx) (164), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX2). Obviously, this positive-feedback loop in ROS-induced p53 activation may be important in achieving a critical threshold of ROS required for the cellular commitment to apoptosis.

V. ROS Regulation of Nuclear Factor of Activated T Cells

The NFAT family members are calcium-sensitive transcription factors originally identified in activated T lymphocytes (165). Five members of the NFAT family have been identified so far. These members include NFAT1 (NFATc2), NFAT2 (NFATc), NFAT3 (NFATc4), NFAT4 (NFATc3), and NFAT5. Despite the fact that subtle functional differences have been found among individual members, all NFAT members require calcineurin (protein phosphatase 2B), a serine/threonine protein phosphatase, for their activation. In resting cells, NFAT is mainly located in the cytoplasm due to its N-terminal serine phosphorylation. Upon exposure to extracellular signals, such as T-cell receptor engagement, a calcium influx is triggered, which activates calcineurin phosphatase through a mechanism dependent on calmodulin. The activated calcineurin dephosphorylates serine residues in the N terminus of NFAT proteins, leading to the unmasking of a nuclear localization sequence and nuclear translocation (166).

A unique structural feature of calcineurin is that its catalytic center contains a binuclear [Fe^{2+}-Zn^{2+}] metal center and four conserved cysteine residues (Cys88, Cys166, Cys197, and Cys228), which are susceptible to redox regulation (167). Indeed, numerous studies suggest that ROS are potent inhibitors of calcineurin (168–177). The pioneering work by Wang et al. demonstrated that the oxidation of Fe by $^{\bullet}O_2^-$ in the Fe-Zn catalytic center results in inactivation of calcineurin (168). Using purified calcineurin, recent studies by Sommer et al. (177) and Bogumil et al. (171) suggest that H_2O_2 inhibits the activity of calcineurin through oxidation of both a catalytic metal and two closely spaced cysteine residues to form a disulfide bond. In agreement with these observations, an inverse relationship between ROS and NFAT transcriptional activity has been demonstrated in several experimental systems (170,178–181).

Based on the above description, it has been generally accepted that oxidative stress due to ROS generation is an inhibitory factor for the

activation of NFAT. However, contradictory observation has also been made (182–184). Huang et al. (182–184) reported that H_2O_2 was responsible for the NFAT activation by metal ions, such as vanadium and nickel, and asbestos. It is unclear what mechanism is involved in this H_2O_2-mediated NFAT activation in the cellular response to metals or asbestos. The authors speculated that ROS might somehow regulate the influx of intracellular calcium ion (183). In Jurkat T cells, the oxidation of cysteine thiols in the calcium release–activated channels was considered to be responsible for the activation of NFAT by vanadium (185).

VI. Summary

ROS generated from eukaryotic cells serve not only as powerful arsenals for the defense of host cells against the invasion of microorganisms but also as critical initiators or regulators for intracellular signal transduction. There is growing body of evidence implicating the association of oxidative stress with a number of human diseases, including cancer, chronic inflammation, and neuronal degeneration. Tremendous advances in our understanding of signal transduction in response to ROS have been achieved in recent years. In addition to PI3K, NF-κB, AP-1, and p53, there are many other intracellular signaling pathways involved in the regulation of and response to ROS. These pathways include Janus protein tyrosine kinases (JAKs) (186), signal transducers and activators of transcription (STATs) (187), phospholipase Cγ (PLCγ) (188), protein kinase C (PKC) (189), focal adhesion kinase (FAK) (190), and hypoxia-inducible factor-1 (HIF-1) (191). Elucidation of the various targets by which the effect of ROS on individual signaling pathways is initiated should provide a rationale for developing therapeutic strategies for amelioration of diseases related to oxidative stress.

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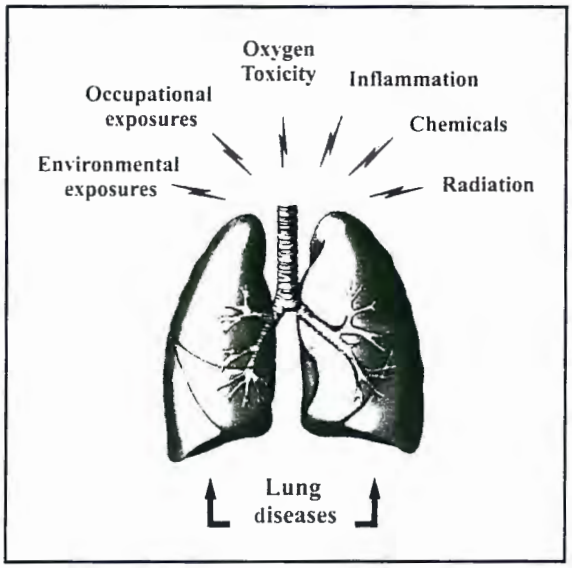
Oxygen/Nitrogen Radicals

Lung Injury and Disease



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