Reactive oxygen species in cell signaling

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Thannickal, Victor J., and Barry L. Fanburg. Reactive oxygen species in cell signaling. Am J Physiol Lung Cell Mol Physiol 279: L1005–L1028, 2000.—Reactive oxygen species (ROS) are generated as by-products of cellular metabolism, primarily in the mitochondria. When cellular production of ROS overwhelms its antioxidant capacity, damage to cellular macromolecules such as lipids, protein, and DNA may ensue. Such a state of “oxidative stress” is thought to contribute to the pathogenesis of a number of human diseases including those of the lung. Recent studies have also implicated ROS that are generated by specialized plasma membrane oxidases in normal physiological signaling by growth factors and cytokines. In this review, we examine the evidence for ligand-induced generation of ROS, its cellular sources, and the signaling pathways that are activated. Emerging concepts on the mechanisms of signal transduction by ROS that involve alterations in cellular redox state and oxidative modifications of proteins are also discussed.

redox signaling; growth factors; cytokines; oxidation-reduction; free radicals

Molecular oxygen (dioxygen; O2) is essential for the survival of all aerobic organisms. Aerobic energy metabolism is dependent on oxidative phosphorylation, a process by which the oxidoreduction energy of mitochondrial electron transport (via a multicomponent NADH dehydrogenase enzymatic complex) is converted to the high-energy phosphate bond of ATP. O2 serves as the final electron acceptor for cytochrome-c oxidase, the terminal enzymatic component of this mitochondrial enzymatic complex, that catalyzes the four-electron reduction of O2 to H2O. Partially reduced and highly reactive metabolites of O2 may be formed during these (and other) electron transfer reactions. These O2 metabolites include superoxide anion (O2·−) and hydrogen peroxide (H2O2), formed by one- and two-electron reductions of O2, respectively. In the presence of transition metal ions, the even more reactive hydroxyl radical (OH·) can be formed. These partially reduced metabolites of O2 are often referred to as “reactive oxygen species” (ROS) due to their higher reactivities relative to molecular O2.

ROS from mitochondria and other cellular sources have been traditionally regarded as toxic by-products of metabolism with the potential to cause damage to lipids, proteins, and DNA (78). To protect against the potentially damaging effects of ROS, cells possess sev-
eral antioxidant enzymes such as superoxide dismutase (which reduces $O_2^-$ to $H_2O_2$), catalase, and glutathione peroxidase (which reduces $H_2O_2$ to $H_2O$). Thus oxidative stress may be broadly defined as an imbalance between oxidant production and the antioxidant capacity of the cell to prevent oxidative injury. Oxidative stress has been implicated in a large number of human diseases including atherosclerosis, pulmonary fibrosis, cancer, neurodegenerative diseases, and aging (56, 103). Yet the relationship between oxidative stress and the pathobiology of these diseases is not clear, largely due to a lack of understanding of the mechanisms by which ROS function in both normal physiological and disease states.

Accumulating evidence suggests that ROS are not only injurious by-products of cellular metabolism but also essential participants in cell signaling and regulation (76, 237). Although this role for ROS is a relatively novel concept in vertebrates, there is strong evidence of a physiological role for ROS in several nonmammalian systems. In bacteria, the OxyR protein functions as a transcriptional regulator of $H_2O_2$-inducible genes and has been shown to be directly activated by oxidation (53, 278). A recent study (324) has shown that $H_2O_2$ oxidizes two conserved cysteines in OxyR to form intramolecular disulfide linkages that trigger the activation of this transcription factor, presumably by changing its conformation. The *Escherichia coli* SoxR transcription factor is activated specifically in response to $O_2^-$-generating redox-cycling agents such as paraquat and menadione (95). Activated SoxR mediates SoxS gene transcription, resulting in an increase in SoxS protein that then activates the transcription of several other genes including superoxide dismutase (SOD) (63). SoxR is a homodimer that contains two redox-active iron-sulfur [2Fe-2S] centers that are sensitive to oxidation by $O_2^-$ (109, 312). The iron-sulfur centers of SoxR must be in their oxidized state for them to be transcriptionally active (85), thus providing a plausible mechanism by which $O_2^-$ transmits its gene regulatory signal. In plant cells, generation of $H_2O_2$ in response to various pathogens elicits localized cell death to limit spread of the pathogen (165) and a more systemic response involving the induction of defense genes regulating plant immunity (10). In sea urchins, fertilization triggers extracellular production of $H_2O_2$ by a plasma membrane oxidase with the simultaneous release of ovoperoxidase (263). Peroxidase- or $H_2O_2$-catalyzed cross-linking of extracellular proteins then forms a protective envelope around the freshly fertilized oocyte.

The apparent paradox in the roles of ROS as essential biomolecules in the regulation of cellular functions and as toxic by-products of metabolism may be, at least in part, related to differences in the concentrations of ROS produced. This is analogous to the effects of nitric oxide (NO), which has both regulatory functions and cytotoxic effects depending on the enzymatic source and relative amount of NO-generated (210). NO functions as a signaling molecule mediating vasodilatation when produced in low concentrations by the constitutive isoform of nitric oxide synthase (NOS) in vascular endothelial cells (309) and as a source of highly toxic oxidants utilized for microbicidal killing when produced in high concentrations by inducible NOS in macrophages (178). Indeed, all phagocytic cells have a well-characterized $O_2^-$-generating plasma membrane oxidase capable of producing the large amounts of ROS required for its function in host defense. In this review, we examine the evidence for the presence of similar plasma membrane oxidases that generate much lower amounts of ROS in nonphagocytic cells for purposes of cell signaling and regulation. We summarize the cellular effects of a wide range of ligand-receptor interactions that have been shown to generate intracellular or extracellular ROS. Emerging concepts on the mechanisms by which ROS may function as signaling molecules are discussed. For purposes of this review, we focus primarily on the cellular effects of ligand-mediated (endogenous) production of ROS rather than on the effects of exogenous oxidative stress. Also, the role of NO as a signaling molecule is not discussed here except in the context of its ability to interact with and modulate $O_2^-$ signaling.

**CHEMISTRY OF ROS**

The biological chemistries of ROS primarily determine the ability of different species to react with specific cellular substrates within the microenvironment in which they are produced. These ROS-substrate reactions are likely to form the basis for our understanding of ROS specificity and their mechanisms of action. ROS have often been “loosely” categorized as free radicals, but this is incorrect because not all ROS are free radicals. A free radical is defined as any atomic or molecular species capable of independent existence that contains one or more unpaired electrons in one of its molecular orbitals (102). Molecular $O_2$ itself qualifies as a free radical because it has two unpaired electrons with parallel spin in different $\pi$-antibonding orbitals. This spin restriction accounts for its relative instability and paramagnetic properties. $O_2$ is capable of accepting electrons to its antibonding orbitals, becoming “reduced” in the process, and, therefore, functioning as a strong oxidizing agent.

A one-electron reduction of $O_2$ results in the formation of $O_2^-$, either by enzymatic catalysis or by “electron leaks” from various electron transfer reactions. $O_2^-$ chemistry in aqueous solution differs greatly from that in organic solvents. In contrast to its remarkable stability in many organic solvents, $O_2^-$ in aqueous solution is short-lived. This “instability” in aqueous solutions is based on the rapid dismutation of $O_2^-$ to $H_2O_2$, a reaction facilitated by higher concentrations of the protonated form of $O_2^-$ ($HO_2^-$) in more acidic pH conditions. Thus the dismutation reaction has an overall rate constant of $5 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ at pH 7.0. SOD speeds up this reaction almost $10^3$-fold (rate constant = $1.6 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$) (79). This implies that any reaction of $O_2^-$ in aqueous solution will be in competition with SOD or, in its absence, with the spontaneous
dismutation reaction itself. NO• reacts with O2• at near diffusion-limited rates and is, therefore, one of the few biomolecules that is able to “outcompete” SOD for O2• (119). Thus in most biological systems, unless sufficiently high concentrations of NO• or other similarly reactive molecules are present, generation of O2• usually results in the formation of H2O2.

Although dismutation of O2• probably accounts for much of the H2O2 produced by eukaryotic cells, H2O2 can also be formed by direct two-electron reduction of O2, a reaction mechanism shared by a number of flavoprotein oxidases (186). Unlike O2•, H2O2 is not a free radical and is a much more stable molecule. H2O2 is able to diffuse across biological membranes, whereas O2• does not. H2O2 is a weaker oxidizing agent than O2•. However, in the presence of transition metals such as iron or copper, H2O2 can give rise to the indiscriminately reactive and toxic hydroxyl radical (OH•) by Fenton chemistry.

CELLULAR SOURCES AND REGULATION OF ROS

Cellular production of ROS occurs from both enzymatic and nonenzymatic sources. As stated earlier, any electron-transferring protein or enzymatic system can result in the formation of ROS as “by-products” of electron transfer reactions. This “unintended” generation of ROS in mitochondria accounts for ~1–2% of total O2 consumption under reducing conditions (78). Due to high concentrations of mitochondrial SOD, the intramitochondrial concentrations of O2• are maintained at very low steady-state levels (298). Thus unlike H2O2, which is capable of diffusing across the mitochondrial membrane into the cytosol (44), mitochondria-generated O2• is unlikely to escape into the cytosol. The potential for mitochondrial ROS to mediate cell signaling has gained significant attention in recent years, particularly with regard to the regulation of apoptosis (25, 40, 46, 154, 166, 302). There is evidence to suggest that tumor necrosis factor (TNF)-α and interleukin (IL)-1-induced apoptosis may involve mitochondria-derived ROS (269, 273, 311). It has also been suggested that the mitochondria may function as an “O2 sensor” to mediate hypoxia-induced gene transcriptions (45, 68).

The endoplasmic reticulum (ER) is another membrane-bound intracellular organelle that, unlike mitochondria, is primarily involved in lipid and protein biosynthesis. Smooth ER (lacking bound ribosomes) contains enzymes that catalyze a series of reactions to detoxify lipid-soluble drugs and other harmful metabolite products. The most extensively studied of these are the cytochrome P-450 and b5 families of enzymes that can oxidize unsaturated fatty acids and xenobiotics and reduce molecular O2 to produce O2• and/or H2O2 (18, 43, 78). Although there does not appear to be a direct link between ER-derived oxidants and growth factor signaling, there is evidence for redox regulation of ER-related functions such as protein folding and secretion (22, 32, 120, 219). Bauskin et al. (32) showed that Ltk, a nonreceptor tyrosine kinase (non-RTK) expressed mainly in lymphocytes, leukemia cells, and neurons, is activated by forming disulfide-linked multimers in response to thiol-oxidizing agents. It has also been suggested that an O2•-generating microsomal NADH oxidoreductase may function as a potential pulmonary artery O2 sensor in pulmonary artery smooth muscle cells (198, 199).

Nuclear membranes contain cytochrome oxidases and electron transport systems that resemble those of the ER but the function of which is unknown (78, 102). It has been postulated that electron “leaks” from these enzymatic systems may give rise to ROS that can damage cellular DNA in vivo (102).

Peroxisomes are an important source of total cellular H2O2 production (37). They contain a number of H2O2-generating enzymes including glycolate oxidase, d-amino acid oxidase, urate oxidase, L-α-hydroxyacid oxidase, and fatty acyl-CoA oxidase. Peroxosomal catalase utilizes H2O2 produced by these oxidases to oxidize a variety of other substrates in “peroxidative” reactions (296). These types of oxidative reactions are particularly important in liver and kidney cells in which peroxisomes detoxify a variety of toxic molecules (including ethanol) that enter the circulation. Another major function of the oxidative reactions carried out in peroxisomes is β-oxidation of fatty acids, which in mammalian cells occurs in mitochondria and peroxisomes (9). Specific signaling roles have not been ascribed to peroxisome-derived oxidants, and only a small fraction of H2O2 generated in these intracellular organelles appears to escape peroxisomal catalase (37, 230).

In addition to intracellular membrane-associated oxidases, soluble enzymes such as xanthine oxidase, aldehyde oxidase, dihydroorotate dehydrogenase, flavoprotein dehydrogenase and tryptophan dioxygenase can generate ROS during catalytic cycling (78). The most extensively studied of these is the O2•-generating xanthine oxidase, which can be formed from xanthine dehydrogenase after tissue exposure to hypoxia (188, 222). Xanthine oxidase is widely used to generate O2• in vitro to study the effect of ROS on diverse cellular processes; however, no studies have implicated a direct physiological role for endogenous xanthine oxidase in cell signaling.

Autooxidation of small molecules such as dopamine, epinephrine, flavins, and hydroquinones can be an important source of intracellular ROS production (78). In most cases, the direct product of such autooxidation reactions is O2•. Although there is no known role for autooxidation of small molecules in growth factor and/or cytokine signaling, such reactions may induce oxidative stress and alter the overall cellular redox state. There is the suggestion that the prooxidant effects of dopamine autooxidation may be involved in the dopamine-induced apoptosis that is implicated in the pathogenesis of neurodegenerative diseases such as Parkinson’s disease (214, 215, 320).

Plasma membrane-associated oxidases have been implicated as the sources of most growth factor- and/or cytokine-stimulated oxidant production (97, 143, 191, 253, 280, 293), although the precise enzymatic sources...
have yet to be fully characterized. The best characterized of the plasma membrane oxidases in general is the phagocytic NADPH oxidase, which serves a specialized function in host defense against invading microorganisms (reviewed in Refs. 20, 258). This multicomponent enzyme catalyzes the one-electron reduction of \( \text{O}_2 \) to \( \text{O}_2^- \), with NADPH as the electron donor through the transmembrane protein cytochrome b\(_{558}\) (a heterodimeric complex of gp91\(_{\text{phox}}\) and p22\(_{\text{phox}}\) protein subunits). The transfer of electrons occurs from NADPH on the inner aspect of the plasma membrane to \( \text{O}_2 \) on the outside. During phagocytosis, the plasma membrane is internalized as the wall of the phagocytic vesicle, with what was once the outer membrane surface now facing the interior of the vesicle. This targets the delivery of \( \text{O}_2^- \) and its reactive metabolites internally for localized microbicidal activity (20).

Recent studies (33, 81, 110, 127, 192, 200) have suggested that functional components of the phagocytic NADPH are present in nonphagocytic cells. Expression of p22\(_{\text{phox}}\) has been demonstrated in the adventitial smooth muscle cells of coronary arteries (19) and the aorta (183). Moreover, polymorphism of the p22\(_{\text{phox}}\) gene has been associated with an increased risk of coronary artery disease in young Caucasian men (84). Increased aortic adventitial \( \text{O}_2^- \) production contributes to hypertension by blocking the vasodilatory effects of NO\(^-\) (305). 3-Hydroxy-3-methylglutaryl-CoA reductase inhibitors appear to have a “cardioprotective” role not only by their effect on lowering cholesterol but also by inhibiting the activity of an \( \text{O}_2^- \)-generating oxidase in vascular endothelial cells and, thereby, improving NO-dependent vasodilation (303). Angiotensin II (ANG II)-induced hypertension is mediated, at least in part, by direct interactions between \( \text{O}_2^- \), generated by a NAD(P)H oxidase, and NO\(^-\) in vascular smooth muscle cells (80). p22\(_{\text{phox}}\) has been shown to be a functional component of this ANG II-stimulated oxidase (183, 322). TNF-\( \alpha \) also stimulates \( \text{O}_2^- \) production in vascular smooth muscle cells by a p22\(_{\text{phox}}\)-based NADH oxidase and appears to upregulate p22\(_{\text{phox}}\) gene expression in these cells (60). Both gp91\(_{\text{phox}}\) and p22\(_{\text{phox}}\) are expressed in pulmonary neuroepithelial cells where a NADPH oxidase-like enzyme may function as an \( \text{O}_2^- \) sensor by activating \( \text{O}_2^- \) and H\(_2\)O\(_2\)-sensitive K\(^+\) channels (304). However, it was later demonstrated that mice with chronic granulomatous disease (lacking a functional gp91\(_{\text{phox}}\) subunit) have preserved hypoxic vasoconstrictive responses and \( \text{O}_2^- \) sensing, suggesting that this oxidase is not involved (14). More recently, Suh et al. (279) demonstrated that Mox-1, a gene encoding a homologue of the catalytic subunit of the phagocytic gp91\(_{\text{phox}}\), is expressed in a number of tissues including vascular smooth muscle. In this study, Mox-1 expression in NIH/3T3 cells was associated with increased \( \text{O}_2^- \) production, serum-stimulated cell growth, and a transformed phenotype. This study suggests that an \( \text{O}_2^- \)-generating oxidase similar (but not identical) to the phagocytic NADPH oxidase is present in some nonphagocytic cells where it functions primarily as a regulator of cellular growth responses.

Cytochrome b\(_{558}\), along with the cytosolic components p67\(_{\text{phox}}\), p47\(_{\text{phox}}\), and p40\(_{\text{phox}}\), makes up the core protein subunits of the phagocytic NADPH oxidase complex. Activation of the oxidase, however, requires the additional participation of Rac2 (or Rac1 in mouse macrophages) and Rap1A, members of the Ras superfamily of small GTP-binding proteins. During activation, Rac2 binds GTP and migrates to the plasma membrane along with the cytosolic components to form the active oxidase complex. A requirement for Rac1 in the activation of the mitogenic oxidase has also been demonstrated in nonphagocytic cells (55, 128). The insert region in Rac1 (residues 124–135) appears to be essential for \( \text{O}_2^- \) production and stimulation of mitogenesis in quiescent fibroblasts but not for Rac1-induced cytoskeletal changes or activation of Jun kinase (128).

Another GTP-binding protein, p21\(_{\text{Ras}}\), appears to function upstream from Rac1 in oxidant-dependent mitogenic signaling (121, 281). Sundaresan et al. (281) showed that dominant negative expression of Rac1 inhibits not only the growth factor- and/or cytokine-generated rise in intracellular ROS in NIH/3T3 cells but also the ROS production in cells overexpressing a constitutively active isoform of Ras (H-Ras\(^{\text{V12}}\)). Stabile transfection of the same Ras plasmid (H-Ras\(^{\text{V12}}\)) in fibroblasts induces cellular transformation and constitutive production of large amounts of \( \text{O}_2^- \) (121). In this study, mitogenic signaling in Ras-transformed fibroblasts was demonstrated to be reduct sensitive but independent of the mitogen-activated protein (MAP) kinase (MAPK) or c-Jun NH\(_2\)-terminal kinase (JNK) pathways. Results from our laboratory (292) demonstrate the requirement for p21\(_{\text{Ras}}\) in the generation of intracellular \( \text{O}_2^- \) by mitochondria such as platelet-derived growth factor (PDGF)-BB, fibroblast growth factor (FGF)-2, and transforming growth factor (TGF)-\( \alpha \) in nontransformed human lung fibroblasts. However, studies from our laboratory (292, 293) also suggest the presence of a distinctly different ROS-generating enzymatic system in these cells that is independent of p21\(_{\text{Ras}}\) regulation and that primarily generates extracellular \( \text{H}_2\text{O}_2 \) in response to TGF-\( \beta 1 \).

In contrast to these relatively recent reports of pyridine- and flavin-linked oxidase activities in nonphagocytic cells, enzymes involved in phospholipid metabolism have been known to exist for several decades. Membrane phospholipids, in addition to their structural role in providing membrane integrity, are substrates for the action of the phospholipases (PLs) PLA\(_2\), PLC, and PLD. Although these enzymes are important for the generation of lipid second messengers, they have generally not been associated with ROS production in nonphagocytic cells. A recent report by Touyz and Schiffrin (297), however, suggests that ANG II-induced \( \text{O}_2^- \) production in smooth muscle cells is dependent on the PLD pathway.

PLA\(_2\) hydrolyzes phospholipids to generate arachidonic acid. Arachidonic acid then forms the substrate for cyclooxygenase- and lipoxygenase (LOX)-dependent reactions.
synthesis of the four major classes of eicosanoids: prostaglandins, prostacyclins, thromboxanes, and leukotrienes. These synthetic pathways involve a series of oxidation steps that involve a number of free radical intermediates (78). Arachidonic acid metabolism, particularly involving the LOX pathway, which leads to leukotriene synthesis, has been reported to generate ROS (30, 170, 207, 272). LOX activity has also been implicated in redox-regulated signaling by ANG II (306), epidermal growth factor (EGF) (196), and IL-1 (36). There is the suggestion that LOX-derived lipid peroxidation products may be involved in the oxidative stress response to asbestos (74). TNF-α-induced apoptosis appears to be mediated by a LOX-dependent but ROS-independent mechanism (213). A lipid-metabolizing enzyme in fibroblasts similar to 15-LOX has been shown to generate large amounts of extracellular O₂⁻ that appears to be independent of flavoenzyme activity (212).

**MEASUREMENT OF ROS IN BIOLOGICAL SYSTEMS**

The high reactivity and relative instability of ROS make them extremely difficult to detect or measure in biological systems. Thus assessments of ROS and free radical generation have largely been made by indirect measurement of various end products resulting from the interaction of ROS with cellular components such as lipids, protein, or DNA (78, 231). Most methods for identification of specific ROS are based on reactions with various “detector” molecules that are oxidatively modified to elicit luminescent or fluorescent signals. Although detailed descriptions of these methods are beyond the scope of this review, we briefly discuss the limitations of the more commonly used assays.

Lucigenin has been used extensively as a chemiluminescent substrate for the detection of O₂⁻ in many biological systems (97, 101, 121, 168, 235, 274). This is based on the ability of lucigenin (Luc²⁺) to be reduced to LucH⁻, which can then react with O₂⁻ to yield an unstable dioxetane compound that yields light (emission of photons) in the process of spontaneously decomposing to its ground-state electronic configuration (73). It has recently been demonstrated (171) that several enzymatic systems in vitro are capable of univalently reducing Luc²⁺ even in the absence of O₂⁻ generation, resulting in the formation of LucH⁻ that can autoxidize to generate O₂⁻. The ability of lucigenin to “redox cycle” in this manner, much like the effects of paraparquat and nitro blue tetrazolium, is thought to be a major limitation in the use of this compound in the detection of O₂⁻ (171). However, the importance of this redox cycling effect of lucigenin when applied to biological systems has been questioned by other investigators (6, 168, 274). The use of an alternative chemiluminescent probe, coelenterazine, may present less of a concern with regard to the potential redox-cycling effects of these compounds (287).

Another technique that is widely used for the detection of intracellular H₂O₂ production in response to growth factor stimulation is based on oxidation of the nonfluorescent substrate 2',7'-dichlorofluorescein (DCFH) to the fluorescent product 2',7'-dichlorofluorescein (DCF) (24, 196, 280). The esterified form of DCFH, dichlorofluorescein diacetate (DCFH-DA), is able to cross cell membranes and then, as a result of deacetylation by intracellular esterases, forms DCFH, which becomes “trapped” intracellularly. This property allows for assessment of intracellular oxidant production. It is becoming increasingly clear, however, that this assay lacks specificity and may be a better indicator of overall changes in the intracellular redox state of the cell than a direct measure of intracellular H₂O₂ production (122, 247). Recent work (244, 245) also suggests that similar to the problem with lucigenin, a series of free radical chain reactions with DCFH in the presence of peroxidase (and even in the absence of H₂O₂) may give rise to “artificial” DCF-dependent fluorescence and O₂ consumption.

Assessments of extracellular ROS production appear to be relatively more reliable and may allow for quantitative measurements. The method for detection of O₂⁻ release with the method of SOD-inhibitable reduction of ferricytochrome c, initially described for neutrophils by Babior et al. (21), has been successfully used in other cell systems (187, 268). However, this method, although quite specific for O₂⁻, lacks sensitivity and may underestimate net O₂⁻ production due to the potential reoxidation of ferricytochrome c by other oxidants (16).

Cellular production of extracellular H₂O₂ release can be measured by a fluorometric method that is based on the ability of heme peroxidases to catalyze H₂O₂-dependent dimerization of substituted phenolic compounds (221, 248). The major advantages of this method are its high specificity and sensitivity for H₂O₂ as determined with the use of horseradish peroxidase (used in most assays). The high sensitivity of such assays is related, at least in part, to the high reactivity of horseradish peroxidase (severalfold higher than catalase) with H₂O₂ (221). We (293) have been able to detect absolute concentrations of H₂O₂ in the 10⁻⁸ M range and a steady-state release of extracellular H₂O₂ of ~22 pmol·min⁻¹·10⁶ cells⁻¹ from TGF-β1-treated fibroblasts in cell culture using this method. Because the fluorescence of the dimerized product is dependent on pH, careful consideration must be made for possible pH changes and overoxidation of the substrate (221).

The only analytic method that, at least theoretically, measures free radicals directly is electron spin resonance spectroscopy. However, it is relatively insensitive and requires steady-state concentrations in the micromolar range for detection and is, therefore, of limited value in most biological systems. This problem may be circumvented by the technique of spin trapping, which involves the addition of a “spin trap” (usually a nitronate or a nitroso compound) that reacts rapidly with free radicals to form more stable radical adducts that accumulate to concentrations in the detectable range (115, 240). Thus all of the currently available methods for the detection of ROS or free radicals in biological systems, including electron spin
resonance spectroscopy, are, by definition, indirect (because detector compounds are required) and semiquan-
titative (because specific ROS are in competition with other biomolecules that can react with these detector molecules). However, if the redox chemistries of the various detector molecules are carefully considered and the potential limitations of these assays are un-
derstood, they can provide useful information on the production and regulation of ROS in biological sys-
tems.

LIGAND-INDUCED ROS PRODUCTION
IN NONPHAGOCYTIC CELLS

A variety of cytokines and growth factors that bind receptors of different classes have been reported to generate ROS in nonphagocytic cells (Table 1). Currently available information on the enzymatic source(s) and physiological actions of ROS generated by these ligand-receptor interactions are summa-
rized here (see Fig. 1 for 3 such pathways).

Cytokine receptors. Cytokine receptors fall into a large and heterogeneous group of receptors that lack intrinsic kinase activity and are not directly linked to ion channels or G proteins. Cytokines such as TNF-α, IL-1, and interferon (IFN)-γ were among the first reported to generate ROS in nonphagocytic cells (187, 190, 191). There is no consensus on the specific species produced, the enzymatic source, or the site of genera-
tion of ROS for these cytokines. In the case of TNF-α, recent reports (49, 169, 257, 270, 319) suggest that a mitochondrial source of ROS is required for activation of the transcription factor nuclear factor (NF)-κB and NF-κB-dependent gene transcription. There is also the suggestion that TNF-α may activate NF-κB by an ROS-
dependent mechanism and that TNF-α and oxidants may produce a synergistic effect in this activation (126). TNF-α-induced generation of mitochondrial ROS has been implicated in apoptotic cell death (269). Over-
expression of Mn SOD inhibits TNF-α-induced apopto-
sis, supporting a role for mitochondrial O$_2^-$ production in mediating this effect (181). The mechanism of TNF-
α-induced apoptosis appears to be related, at least partially, to depletion of GSH, leading to redox-depen-
dent formation of ceramide from sphingomyelin (172, 273). Gotoh and Cooper (94) showed that TNF-α acti-
vates apoptosis signal-regulating kinase-1 (ASK-1), a member of the MAPK kinase superfamily, by inducing oxidant-dependent dimerization of ASK-1. The resistance to apoptosis in some cancer cells may be related to the constitutive activation of NF-κB by au-
tocrine production of TNF-α (86). ROS-dependent mechanisms also appear to be involved in TNF-α-in-
duced expression of cell adhesion molecules (12, 147, 233, 318), IL-8 expression (147, 266), production of chemokines (27, 62), and induction of cardiac myocyte hypertrophy (206).

Many of the redox-regulated effects of IL-1 on cell function appear to be similar to those of TNF-α. Both IL-1 and TNF-α have been implicated in playing a role in the cardioprotective effects of exercise by upregulat-
ing Mn SOD in a rat model of ischemia-reperfusion (316). These cytokines also upregulate heme oxygen-
ase-1 in endothelial cells by redox-dependent mecha-
nisms that involve protein kinase (PK) C, intracellular Ca$^{2+}$, and PLA$_2$ (289). IL-1, TNF-α, and H$_2$O$_2$ have been shown to induce insulin-like growth factor binding protein-1 synthesis in Hep G2 cells (152). In this study, the effect of cytokines, although sensitive to pyrroldine dithiocarbamate, appears to be independent of endogenous ROS production. Bonizzi et al. (36) recently demonstrated three different cell-specific pathways leading to NF-κB activation by IL-1: a path-
way dependent on ROS production by 5-LOX in lymphoid cells, a ROS- and 5-LOX-independent pathway in epithelial cells, and a pathway requiring ROS pro-
duction by NADPH oxidase in monocytic cells. ROS-
dependent activation of NF-κB by IL-1β in epithelial cells appears to involve the acidic sphingomyelinase/
ceramide transduction pathway (228). There is evidence for flavoenzyme-generated ROS in the induction of c-Fos and collagenase expression by IL-1 in chondro-
cytes (175). Expression of collagenase by IL-1 may be mediated by an autocrine loop involving Rac1, ROS, and NF-κB in fibroblasts (136). IL-1 may inhibit fibrinolysis and contribute to vascular injury by inducing plasminogen activator inhibitor type 1 and type 1 collagen expression in a ROS-dependent manner in rat cardiac microvascular endothelial cells (217).

IFN-γ is well recognized as an activator of the phago-
cytic NADPH oxidase that has an important role in defense against pathogenic bacteria (21). A recent re-
port (146) suggests that IFN-γ stimulates cyclooxyge-
nase-dependent peroxide production in murine and human hepatocytes that confers resistance against bacterial growth. In contrast, a ROS-independent mechanism of endothelial cytoprotection from Candida albicans infection also appears to be mediated by IFN-γ (77).

RTKs. A number of growth factors that bind RTKs have been shown to generate intracellular ROS essen-
tial for mitogenic signaling. A decade ago, PDGF was shown to stimulate cellular production of H$_2$O$_2$, which was thought to function as a “competence factor” in BALB/3T3 cell growth (264). Sundaresan et al. (280) demonstrated the ability of PDGF to transiently in-
crease intracellular concentrations of H$_2$O$_2$, which was required for PDGF-induced tyrosine phosphorylation, MAPK activation, DNA synthesis, and chemotaxis. PDGF also activates the signal transducer and activa-
tor of transcription (STAT) family of transcription fac-
tors by a H$_2$O$_2$-dependent mechanism (271). Interest-
ingly, H$_2$O$_2$ appears to antagonize the PDGF effect on disruption of gap junction communication by abrogat-
ing the phosphorylation of a gap junctional protein, connexin 43, by a tyrosine kinase- and redox-depen-
dent mechanism (116). PDGF has also been shown to regulate gene expression by O$_2^-$-dependent pathways. PDGF stimulates flavoenzyme-dependent O$_2^-$ genera-
tion in human aortic smooth muscle cells by PKC- and wortmannin-sensitive pathways (184). In this study, PDGF-induced O$_2^-$ production appears to participate
Table 1. Ligand-mediated generation of ROS in nonphagocytic cells

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Reactive Species</th>
<th>Enzymatic Source(s)</th>
<th>Cell or Tissue</th>
<th>Functional (or Pathophysiological) Effects</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>O₂⁻, H₂O₂</td>
<td>NADP/H oxidase</td>
<td>Fibroblasts</td>
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<td>Endothelial cells</td>
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<td>202</td>
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<tr>
<td></td>
<td>O₂⁻</td>
<td>NADPH oxidase</td>
<td>Mesangial cells</td>
<td>MCP-1, CSF-1 expression</td>
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</tr>
<tr>
<td></td>
<td>H₂O₂</td>
<td>Flavoprotein oxidase</td>
<td>Chondrocytes</td>
<td>Mitogenesis</td>
<td>176</td>
</tr>
<tr>
<td>Unspecified</td>
<td>Mitochondria</td>
<td>Fibrosarcoma</td>
<td></td>
<td>NFP-α activation, IL-6 induction</td>
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</tr>
<tr>
<td></td>
<td>Unknown</td>
<td>Breast carcinoma cells</td>
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<td>HSP27 phosphorylation</td>
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<tr>
<td>+ IL-1</td>
<td>Unspecified</td>
<td>Flavoprotein oxidase</td>
<td>Rat mesangial cells</td>
<td>COX-2 expression</td>
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<tr>
<td>+ IFN-γ</td>
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<td>Microglial cells</td>
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<tr>
<td>IL-1</td>
<td>O₂⁻, H₂O₂</td>
<td>NADP/H oxidase</td>
<td>Fibroblasts</td>
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</tr>
<tr>
<td>O₂⁻</td>
<td>Unknown</td>
<td>Endothelial cells</td>
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<td>187</td>
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<tr>
<td>O₂⁻</td>
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<td>Dog myocardium</td>
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<tr>
<td>IFN-γ</td>
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<td>Endothelial cells</td>
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<tr>
<td>H₂O₂</td>
<td>Cyclooxygenase</td>
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Receptor tyrosine kinases

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<th>Functional (or Pathophysiological) Effects</th>
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<tbody>
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<td>PDGF</td>
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<td>BALB/3T3 cells</td>
<td>&quot;Competence factor&quot; for cell growth</td>
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<tr>
<td>H₂O₂</td>
<td>Flavoprotein oxidase</td>
<td>Smooth muscle cells</td>
<td>Mitogenesis, MAPK activation</td>
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<tr>
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<td>Unknown</td>
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<td>NOS expression, PGE₂ release</td>
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<td>Flavoenzyme</td>
<td>Smooth muscle cells</td>
<td>NFP-α-dependent MCP-1 induction</td>
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<td></td>
</tr>
<tr>
<td>EGF</td>
<td>H₂O₂</td>
<td>Unknown</td>
<td>Epidermoid carcinoma</td>
<td>Tyrosine phosphorylation, cell growth</td>
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<tr>
<td>Unspecified</td>
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<td>JNK activation</td>
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<tr>
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<td>Hepatocytes</td>
<td>Carcinogenesis</td>
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<tr>
<td>Unspecified</td>
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<td>Human keratinocytes</td>
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<tr>
<td>O₂⁻</td>
<td>Lipoxygenase</td>
<td>PC12</td>
<td>Cell growth</td>
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<td>H₂O₂</td>
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<td>Rat mammary CA cells</td>
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<tr>
<td>H₂O₂</td>
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<td>Mouse epidermal cells</td>
<td>Human growth</td>
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<td>HB-EGF</td>
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<td>Unknown</td>
<td>Smooth muscle cells</td>
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<td>FGF-2</td>
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<tr>
<td>O₂⁻</td>
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<td>Lung fibroblasts</td>
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<td>292</td>
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<tr>
<td>IGF-1</td>
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<td>STS 1-pseudopocytes</td>
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<tr>
<td>HGF</td>
<td>H₂O₂</td>
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Receptor serine/threonine kinases

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<tr>
<td>TGF-β1</td>
<td>H₂O₂</td>
<td>Unknown</td>
<td>Mouse osteoblasts</td>
<td>Growth inhibition; erg-1 induction</td>
<td>216, 265</td>
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<tr>
<td></td>
<td>H₂O₂</td>
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<td>Endothelial cells</td>
<td>Growth inhibition</td>
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<td>H₂O₂</td>
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<td>Unknown</td>
<td>Rat hepatocytes</td>
<td>Growth inhibition</td>
<td>132</td>
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<td>H₂O₂</td>
<td>NADH oxidase</td>
<td>Lung fibroblasts</td>
<td>Unknown</td>
<td>280, 293</td>
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<tr>
<td>H₂O₂</td>
<td>Unknown</td>
<td>Hepatocytes</td>
<td>Apoptosis</td>
<td>251, 252</td>
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<tr>
<td>H₂O₂</td>
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<td>Unknown</td>
<td>Pancreatic beta cells</td>
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<tr>
<td>H₂O₂</td>
<td>Unknown</td>
<td>Hepatic stellate cells</td>
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<td>59, 83</td>
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G protein-coupled receptors

<table>
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<tr>
<th>Ligand</th>
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<th>Enzymatic Source(s)</th>
<th>Cell or Tissue</th>
<th>Functional (or Pathophysiological) Effects</th>
<th>Reference No.</th>
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</thead>
<tbody>
<tr>
<td>Angiotensin II</td>
<td>O₂⁻, H₂O₂</td>
<td>NADP/H oxidase, p70Gαq</td>
<td>Smooth muscle cells</td>
<td>Cell hypertrophy, p38 activation</td>
<td>97, 301, 322</td>
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<td></td>
<td>O₂⁻</td>
<td>Unknown</td>
<td>Proximal tubule cells</td>
<td>g70Gαq-1, cell hypertrophy</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>H₂O₂</td>
<td>Flavoprotein oxidase</td>
<td>Mesangial cells</td>
<td>Cell hypertrophy</td>
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<tr>
<td></td>
<td>PLD-dependent</td>
<td>Smooth muscle cells</td>
<td>Proliferation/hypertrophy</td>
<td>297</td>
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<tr>
<td>Serotonin</td>
<td>O₂⁻</td>
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<td>5-HT transporter</td>
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<td>Cyclooxygenase</td>
<td>Endothelial cells</td>
<td>Unknown</td>
<td>114, 282</td>
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<tr>
<td></td>
<td>O₂⁻</td>
<td>Unknown, Ca²⁺</td>
<td>Human keratinocytes</td>
<td>Unknown</td>
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<tr>
<td>Thrombin</td>
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<td>NADP/H-like enzyme</td>
<td>Endothelial cells</td>
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<tr>
<td></td>
<td>O₂⁻, H₂O₂</td>
<td>NADP/H oxidase, p42Gα</td>
<td>Smooth muscle cells</td>
<td>Cell growth, p38 activation</td>
<td>223, 236</td>
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<tr>
<td>Endothelin</td>
<td>H₂O₂</td>
<td>Unknown</td>
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Ion channel-linked receptors

<table>
<thead>
<tr>
<th>Ligand</th>
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<th>Enzymatic Source(s)</th>
<th>Cell or Tissue</th>
<th>Functional (or Pathophysiological) Effects</th>
<th>Reference No.</th>
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<tr>
<td>Glutamate</td>
<td>H₂O₂</td>
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<td>Rat cerebral cortex</td>
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<tr>
<td>O₂⁻</td>
<td>Mitochondria</td>
<td>Neurons</td>
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<td>261</td>
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<tr>
<td>Acetylcholine</td>
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<td>Mitochondria, Zn²⁺</td>
<td>Cardiomyocytes</td>
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<td>317</td>
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</table>

ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; MCP-1, monocyte chemoattractant protein-1; CSF-1, colony-stimulating factor-1; NF-κB, nuclear factor-κB; IL, interleukin; HSP27, 27-kDa heat shock protein; COX-2, cyclooxygenase-2; IFN-γ, interferon-γ; PDGF, platelet-derived growth factor; MAPK, mitogen-activated protein kinase; NOS, nitric oxide synthase; EGF, epidermal growth factor; JNK, c-Jun NH₂-terminal kinase; PLA₂, phospholipase A₂; HB-EGF, heparin-bound EGF; FGF-2, fibroblast growth factor-2; IGF-1, insulin growth factor I; HGF, hepatocyte growth factor; TGF, transforming growth factor; AOE, antioxidative enzyme; PLD, phospholipase D; 5-HT, 5-hydroxytryptamine (serotonin); ERK, extracellular signal-regulated kinase.
in vascular lesion formation by activating NF-κB and inducing monocyte chemoattractant protein-1 expression. PDGF-induced O$_2^-$ (but not H$_2$O$_2$) appears to be involved in its upregulation of inducible NOS- and NO$_2^-$-dependent release of PGE$_2$ in fibroblasts (134).

Bae et al. (24) demonstrated that EGF-induced intracellular H$_2$O$_2$ formation in A547 cells requires EGF receptor kinase (but not the autophosphorylation sites on the EGF receptor) and that inhibition of protein tyrosine phosphatase activity by H$_2$O$_2$ is required for EGF-induced protein tyrosine phosphorylation. EGF has been shown to stimulate a rise in intracellular Ca$^{2+}$ levels in a keratinocyte cell line followed by an increase in intracellular ROS and PLA$_2$ activation (89, 90). In PC12 cells, EGF stimulates intracellular O$_2^-$ production, involving the LOX pathway, and this is attenuated by pretreatment of cells with nerve growth factor by a NO-dependent mechanism (196). EGF-generated ROS were found to mediate inhibitory cross talk with a signaling pathway activated by a G protein-coupled receptor (323). Recently, hepatocyte growth factor (scatter factor) has also been shown to stimulate ROS in a sarcoma cell line.

Basic FGF (FGF-2) has been shown to stimulate a flavoprotein oxidase that generates the intracellular H$_2$O$_2$ required for the induction of c-Fos in chondrocytes (176). Our laboratory has shown that FGF-2 (in addition to other RTK-linked mitogens) generates O$_2^-$ in human lung fibroblasts (292). In this study, O$_2^-$ (preferentially over H$_2$O$_2$) appears to mediate a common mitogenic signaling by a number of RTK(s)-linked growth factors through a p38 MAPK-dependent pathway. Recently, hepatocyte growth factor (scatter factor) has also been shown to stimulate ROS in a sarcoma cell line (ER-1) have also been shown to be related, at least partially, to EGF-induced peroxide production (204).
line where it appears to mediate an apoptotic, as opposed to a mitogenic, response (13).

Several RTK-linked growth factors have also been shown to have inhibitory effects on cellular ROS production. Heck et al. (106) showed that EGF downregulates the LPS- and cytokine-mediated H$_2$O$_2$ and NO production in primary skin keratinocytes. Different members of the PDGF and FGF families, in an isoform-specific manner, appear to have either stimulatory or inhibitory effects on the activation of a H$_2$O$_2$-generating NADPH oxidase that has a role in adipocyte differentiation of 3T3 L1 cells (143). FGF-2 has also been shown to be neuroprotective by inhibiting β-amyloid-induced ROS accumulation in hippocampal neurons (182). A similar neuroprotective effect is seen with nerve growth factor, which suppresses ROS production by a MAPK-dependent mechanism in a central nervous system neuronal cell line (67). These apparent opposing effects on ROS production may be related to tissue- and growth factor-specific actions.

Receptor serine/threonine kinases. All receptor serine/threonine kinases described to date in mammalian cells are members of the TGF-β superfamily. TGF-β1 is the prototype of this large family of polypeptide growth factors that bind receptor serine/threonine kinases and include the TGF-βs, activins, inhibins, bone morphogenetic proteins, and Mullerian-inhibiting substance (185, 227). Each member of the TGF-β superfamily activates a heteromeric complex of type I-type II receptors in a combinatorial manner, leading to the phosphorylation and/or activation of the recently described SMAD proteins that translocate to the nucleus to mediate gene transcription (185). Unlike RTK(s)-linked growth factors, TGF-β1 typically inhibits growth of most target cells. Proliferative responses to TGF-β1 appear to be primarily related to the indirect effects on the autocrine production of mitogenic growth factors (29, 164) or their receptors (241, 291). These multifunctional effects of TGF-β on cellular growth regulation as well its diverse effects on cell differentiation and extracellular matrix production are critical in complex biological processes such as embryogenesis, fibrosis, and carcinogenesis.

TGF-β1 has been shown to stimulate ROS production in a variety of cell types (123, 132, 251, 264, 293, 294). Shibanuma et al. (265) demonstrated a cell cycle-dependent extracellular release of H$_2$O$_2$ (only during the late G$_1$ and not the G$_0$ phase) from mouse osteoblastic cells stimulated with TGF-β1 in association with phosphorylation of a 30-kDa protein. It was proposed from this study that H$_2$O$_2$ was mediating the growth-inhibitory effects of TGF-β1. Another study from the same laboratory (216) examined intracellular ROS production in the same cell line and its role in the induction of the early growth response-1 (egr-1) gene by TGF-β1. In this study, extracellular catalase blocked both the TGF-β1-induced increase in intracellular H$_2$O$_2$ and egr-1 gene expression, leading the authors to propose that H$_2$O$_2$ may be released extracellularly before diffusing back into the cell. Studies from our laboratory (293, 294) support the concept that TGF-β1-stimulated H$_2$O$_2$ production is generated primarily extracellularly. In human lung fibroblasts, this effect appears to be mediated by activation of a flavoenzyme with cell surface-associated NADH:flavin:O$_2$ oxidoreductase activity that is regulated by protein tyrosine phosphorylation (292, 294). More recent evidence (308) suggests that the assembly and activation of this oxidase complex require Src kinase(s) activation and actin cytoskeletal regulation.

TGF-β1 has also been shown to suppress the expression of antioxidant enzymes in some cells (123, 132). Kayanoki et al. (132) showed that TGF-β1 inhibits the expression of Mn SOD, copper-zinc SOD, and catalase in rat hepatocytes, leading to increased cellular oxidative stress. Arsalane et al. (15), Das et al. (58), and White et al. (308) have shown that TGF-β1 lowers cellular concentrations of intracellular glutathione (GSH) in some lung cells, primarily endothelial and epithelial cells. This appears to be mediated by an inhibitory effect of TGF-β1 on transcription of the rate-limiting enzyme involved in GSH synthesis, γ-glutamylcysteine synthetase (15). The reduction in cellular GSH levels by TGF-β1 in vascular endothelial cells is closely associated with its growth-inhibitory effect and appears to be modulated by thiol availability (58). A recent study (59) suggests that intracellular levels of GSH may be important in discriminating an oxidative stress from a “signaling” response to TGF-β1. In this study, endogenously generated H$_2$O$_2$ appears to mediate TGF-β1 autoinduction but only under conditions in which intracellular GSH concentrations are high.

The specific molecular targets of TGF-β1-stimulated ROS are unknown, but its prooxidant effect on various cells has been proposed to regulate a number of physiological actions. These include its growth-inhibitory effects (58, 265), apoptosis (31, 105, 123, 145, 153, 251, 252), TGF-β1 autoinduction (59), activation of latent TGF-β (26), cellular transformation (129), collagen synthesis (83) and myofibroblast differentiation (59, 155).

G protein-coupled receptors. G protein-coupled receptors are the largest family of cell surface receptors, with >100 members characterized in mammals (9, 239). A number of ligands that bind to these receptors have been shown to generate ROS in different cell systems. Examples of these ligands include ANG II, serotonin [5-hydroxytryptamine (5-HT)], bradykinin, thrombin, and endothelin (ET).

ANG II is a vasoactive peptide that in addition to its effect on vasomotor tone, has proliferative and hypertrophic effects on vascular smooth muscle. ANG II has been shown to stimulate ROS production in cultured vascular smooth muscle cells (97, 297), glomerular mesangial cells (125), endothelial cells (288) and renal proximal tubular cells (104). Griendling et al. and Zafari et al. demonstrated that ANG II activates both NADH- and NADPH-dependent O$_2$- production in vascular smooth muscle cells (97) and that this activity requires the functional expression of p22phox (322). Physiological actions of ANG II that appear to be mediated by ROS include its vasopressor activity (235),
smooth muscle cell hypertrophy (322), activation of cell survival PK Akt/PKB (300), induction of insulin-like growth factor-1 receptor (66), and IL-6 production (142). A recent study by Ushio-Fukai et al. (299) suggests that ANG II stimulates, in parallel, both p42/p44 and p38 MAPK pathways along with the generation of intracellular H2O2. However, in this study, only the p38 pathway was oxidant sensitive, and simultaneous inhibition of both pathways was required to block ANG II-induced cell hypertrophy, suggesting that there is cross talk between redox-sensitive and -insensitive MAPK pathways in mediating this effect.

5-HT, similar to ANG II, is a vasoactive peptide that mediates both cellular hypertrophy and hyperplasia in vascular smooth muscle cells (72, 160) while functioning as a neurotransmitter in neuronal cells. Although 5-HT binds to a number of different G protein-coupled receptors and other less well-characterized 5-HT receptors (9, 34), work from our laboratory (156, 161) suggests that its mitogenic effect on vascular smooth muscle cells is primarily related to the internalization of 5-HT through a 5-HT transporter protein. Intracellular transport of 5-HT induces tyrosine phosphorylation of 5-HT transporter and receptor activation, the generation of 5-HT-porter and receptor activation), the generation of 5-HT-induced O2•− leads to the activation of the p42/p44 MAPK pathway and cellular proliferation. A study by Grewal et al. (96) in mesangial cells suggests that a similar signaling pathway via the 5-HT transporter mechanism and Chinese hamster lung fibroblasts (via both 5-HT transporter and receptor activation), the generation of 5-HT-induced O2•− leads to the activation of the p42/p44 MAPK pathway and cellular proliferation. A study by Sobey et al. (275) suggests that the vasodilator responses of cerebral arterioles to bradykinin involve ROS-mediated activation of Ca2+-dependent K+ channels. Similar findings invoking a potential role for OH•-bradykinin-induced vasodilation of cerebral arterioles have been previously reported (242). However, a recent report (229) also suggests that an ROS-independent mechanism may mediate this effect.

Thrombin and ET-1 are potent vascular smooth muscle cell mitogens that also signal via G protein-coupled receptors (239). Thrombin has been shown to generate ROS in both endothelial cells (113) and smooth muscle cells (223, 236). Patterson et al. (223) recently demonstrated the ability of thrombin to stimulate O2•− and H2O2 production in vascular smooth muscle cells that is associated with the NAD(P)H oxidase activity required for thrombin-induced mitogenesis (223). In this study, thrombin increased expression of both p47phox and Rac2 in these cells and stimulated their translocation to the membrane, suggesting that an oxidase similar to the phagocytic NADPH oxidase is involved. In another study by Rao et al. (236), redox-dependent mitogenic signaling by thrombin in smooth muscle cells appears to involve extracellular signal-regulated kinase (ERK) 2, JNK1, and p38 MAPKs (because agonist-induced activation of all pathways was inhibited by N-acetylcysteine). However, in this study, only a subset of the early signaling events was dependent on oxidation because p38 MAPK and c-Fos/Jun B but not other pathways were inhibited by the flavoprotein inhibitor diphenyleneiodonium. Recently, ET-1 was also demonstrated to increase intracellular ROS production in cardiac myocytes by a p21Ras-dependent mechanism (50). In this study, the ET-1-induced ROS were essential for the induction of c-Fos expression. In another study, ROS also appear to mediate the inotropic effects of ET-1 on the myocardium (61).

**Ion channel-linked receptors.** Ion channel-linked receptors mediate rapid synaptic signaling between electrically excitable cells by transiently opening and closing an ion channel formed by the ligand-bound receptor complex. Such ligands typically are neurotransmitters (e.g., acetylcholine, 5-HT, glutamate, glycine, and γ-aminobutyric acid). Compared with the other classes of receptors discussed above, relatively little is known about ROS signaling by ion channel-linked receptors. However, there is growing interest in the role of ROS in mediating neuronal cell death by some excitatory neurotransmitters. Glutamate induces ROS production in neuronal cells by mechanisms that have been shown to be both dependent and independent of the changes in intracellular ion concentrations (35, 260). Work by Sensi and colleagues (260, 261) suggests that ROS generation in these cells is a result of intracellular Zn2+-mediated mitochondrial depolarization. Acetylcholine has been shown to activate ATP-sensitive K+ channels and increase mitochondrial ROS that may function as an intracellular signal for acetylcholine-induced preconditioning in cardiomyocytes (317).

**SIGNALING MOLECULES “TARGETED” BY ROS**

Although a large number of signaling pathways appear to be regulated by ROS, the signaling molecules targeted by ROS are less clear. There is growing evidence, however, that redox regulation might occur at multiple levels in the signaling pathways from receptor to nucleus.

Receptor kinases and phosphatases themselves may be targets of oxidative stress. Growth factor receptors are most commonly activated by ligand-induced dimer-
ization or oligomerization that autophosphorylates its cytoplasmic kinase domain (107). Ligand-independent clustering and activation of receptors in response to ultraviolet light have also been well demonstrated (243, 249), and this effect appears to be mediated by ROS (118, 226). Exogenous H$_2$O$_2$ (usually in the millimolar range) has been shown to induce tyrosine phosphorylation and activation of the PDGF-α, PDGF-β, and EGF receptors (82, 87, 91). Lysophosphatidic acid-induced activation of the EGF receptor appears to be mediated by the intermediate formation of ROS (57). A study by Knebel et al. (138) suggests that the mechanism of these effects may be related to ROS-mediated inhibition of the dephosphorylation of RTKs by inactivation of membrane-bound protein tyrosine phosphatases(s). A similar action of either O$_2^\cdot$- or NO-generating stimuli on the phosphorylation-dephosphorylation balance leading to PDGF receptor activation has also been reported (41). However, to our knowledge, no studies have demonstrated the ability of endogenous, growth factor-stimulated ROS to regulate its own (or other) receptor(s) activation. On the other hand, H$_2$O$_2$ production in response to EGF has been shown to require a functional EGF receptor kinase domain (24), suggesting that growth factor-induced ROS may function downstream from EGF receptor activation. This does not exclude the possibility that, under certain pathological conditions associated with oxidative stress, ROS may directly activate cell surface receptors.

Because most growth factors and cytokines appear to generate ROS at or near the plasma membrane, phospholipid metabolites are potentially important targets for redox signaling. Takekoshi et al. (286) showed that the oxidized forms of diacylglycerol were more effective in activating PKC than its nonoxidized forms. PKC activation and protein tyrosine phosphorylation appear to be required for H$_2$O$_2$-induced PLD activation in endothelial cells and fibroblasts (197, 209). This effect may be mediated by the upstream activation of growth factor receptors because suramin (an inhibitor of receptor activation) blocks H$_2$O$_2$-induced PLD activation (208). EGF signaling of PLA$_2$ activation and arachidonic acid release is sensitive to antioxidants suggesting that PLA$_2$ may be a target of ROS (88).

Non-RTKs belonging to the Src family (Src kinases) and Janus kinase (JAK) family have been reported to be regulated by various forms of oxidative stress (2, 7, 271). As in the case of RTKs, these reports relate primarily to the effects of exogenously added oxidants, and the significance of such actions in specific growth factor- or cytokine-mediated signaling is less clear. Simon et al. (271) have demonstrated, however, that PDGF-induced activation of the JAK-STAT pathway is redox sensitive, suggesting a role for endogenous oxidant production in mediating this effect. Src kinases and JAKs appear to be involved in H$_2$O$_2$-induced activation of p21$^{Ras}$ in fibroblasts (2). In another study, Lander et al. (150) have shown that p21$^{Ras}$ may be a common signaling target of NO- and ROS. This effect may involve the recruitment of phosphatidylinositol 3'-kinase to the plasma membrane where it associates directly with the effector domain of p21$^{Ras}$ before being activated (64).

ROS signaling by the induction of changes in intracellular Ca$^{2+}$ ([Ca$^{2+}$]) has been studied in a number of cell types including smooth muscle cells (reviewed in Ref. 285). Roveri et al. (246) showed that H$_2$O$_2$ induces a rapid increase in [Ca$^{2+}$], that appears to be related to inositol 1,4,5-triphosphate-sensitive Ca$^{2+}$ stores in the sarcoplasmic reticulum (SR) followed by a slower increase in [Ca$^{2+}$] that is most likely derived from the extracellular space (246). Both O$_2^\cdot$ (98, 283, 284) and H$_2$O$_2$ (99) have been shown to inhibit the activity of ATP-dependent Ca$^{2+}$ of the SR, which would result in passive diffusion of SR Ca$^{2+}$ into the cytosol. Such effects may also be more important in oxidative stress responses (179) than in receptor-mediated signaling by growth factors and/or cytokines.

The MAPKs comprise a large family of PKs that include ERK1 (p44MAPK)/ERK2 (p42MAPK), JNKs (also known as the stress-activated PKs), and p38 MAPKs. Because the MAPK pathways mediate both mitogen- and stress-activated signals, there has been significant interest in the redox regulation of these pathways. A number of groups (4, 23, 100, 195, 201, 277) have demonstrated the ability of exogenous oxidants to activate the ERK MAPK pathway. The mechanism(s) for this effect is unclear, and the precise molecular target(s) is unknown. Some studies suggest that ROS-mediated ERK activation may be an upstream event at the level of growth factor receptors (100), Src kinases (7), and/or p21$^{Ras}$ (201). Another potential mechanism for this effect may be oxidant-induced inactivation of protein tyrosine phosphatases (PTPs) and/or protein phosphatase A (307). There are relatively fewer reports of endogenous ROS regulating the ERK MAPK pathway. However, Wilmer et al. (310) showed that IL-1-induced activation of ERK2 and JNK in human mesangial cells was inhibited by antioxidants, suggesting that ligand-stimulated ROS may be involved in mediating this effect. Also, serum- and 12-O-tetradecanoylphorbol-13-acetate-induced p21 induction appears to be mediated by redox-regulated ERK activation in HeLa cells (70). Results from our laboratory (159) suggest that redox regulation of the ERK pathway may be ligand and cell specific. 5-HT-induced ERK1/ERK2 activation in smooth muscle cells is redox sensitive, whereas FGF-2-stimulated activation of ERK1/ERK2 in lung fibroblasts is not (Than Nickel et al., unpublished observations). Recent work also suggests that cyclin D1 expression, independent of the ERK MAPK pathway, may be a downstream target of redox-regulated mitogenic signaling (218). In vascular endothelial cells, cyclic strain-induced ROS can modulate egr-1 expression, at least partially, via the ERK signaling pathway (314).

Other members of the MAPK family have also been implicated as potential targets of ROS. Big MAPK-1 (BMK-1) appears to be much more sensitive than ERK1/ERK2 to H$_2$O$_2$ in several cell lines tested and suggests a potentially important role for BMK-1 as a
MECHANISMS OF ROS ACTION

Current concepts of ROS signaling can be divided into two general mechanisms of action: 1) alterations in intracellular redox state and 2) oxidative modifications of proteins.

Alterations in intracellular redox state. In comparison with the extracellular environment, the cytosol is normally maintained under strong “reducing” conditions. This is accomplished by the “redox-buffering” capacity of intracellular thiols, primarily GSH and thioredoxin (TRX). The high ratios of reduced to oxidized GSH and TRX are maintained by the activity of GSH reductase and TRX reductase, respectively. Both of these thiol redox systems counteract intracellular oxidative stress by reducing both H2O2 and lipid peroxides, reactions that are catalyzed by peroxidases (e.g., GSH peroxidase catalyzes the reaction 
\[ H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG \]). Accumulating evidence suggests that, in addition to their “antioxidant” functions, GSH and TRX participate in cell signaling processes.

GSH has been reported to regulate redox signaling by alterations in both the level of total GSH (65, 69, 259) and in the ratio of its oxidized (GSSG) to reduced (GSH) forms (108, 139). Cellular GSH depletion has been found to be associated with decreased cell proliferation in vascular endothelial cells (58, 308) and increased proliferation of fibroblasts (42). TGF-β1-induced growth inhibition of vascular endothelial cells is accompanied by GSH depletion, and repletion of intracellular thiols, at least partially, reverses its antiproliferative effect (58). Rigacci et al. (238) showed that autophosphorylation of the PDGF receptor is inhibited by low cellular GSH levels. In the nucleus, the GSH redox couple appears to regulate DNA binding of the Sp-1 transcription factor (139).

TRX is a small multifunctional protein with two redox-active cysteines within a conserved active site (Cys-Gly-Pro-Cys) (reviewed in Ref. 205). Recent evidence suggests that TRX can regulate the activity of some proteins by directly binding to them. Saitoh et al. (250) have shown that TRX is an inhibitor of ASK-1 by binding to its amino-terminal domain and that both TNF-α and ROS activate ASK-1 by inducing dissociation of TRX. Recent work by Liu et al. (173) suggests that the dissociation of TRX is followed by the binding of TNF receptor-associated factor-2 (TRAF-2) to ASK-1 and consequent ASK-1 multimerization. Interactions similar to those described between TRX and ASK-1 have been found with other signaling molecules as well. A mammalian two-hybrid assay revealed direct association between TRX and the glucocorticoid receptor, and this interaction was favored under oxidative conditions within the nucleus (180). There is also evidence that TRX can translocate from the cytosol to the nucleus in response to oxidant stress to regulate gene expression through Ref-1. Binding and activation of Ref-1 by TRX facilitates DNA binding of the Jun-Fos complex to the AP-1 site to mediate transcription (111). A similar effect of TRX on DNA binding of NF-κB has also been demonstrated (112). Other transcription factors that are regulated by TRX include p53 (225) and polyomavirus enhancer binding protein 2/core binding factor (PEBP2/CBF), which contains two conserved redox-sensitive cysteines in its Runt domain (8).
Oxidative modifications of proteins. ROS can alter protein structure and function by modifying critical amino acid residues, inducing protein dimerization, and interacting with Fe-S moieties or other metal complexes (see Fig. 2). Oxidative modifications of critical amino acids within the functional domain of proteins may occur in several ways. By far, the best described of such modifications involves cysteine residues. The sulfhydryl group (-SH) of a single cysteine residue may be oxidized to form sulfenic (-SOH), sulfinic (-SO2H), sulfonic (-SO3H), or S-glutathionylated (-SSG) derivatives (Fig. 2A). Such alterations may alter the activity of an enzyme if the critical cysteine is located within its catalytic domain (28) or the ability of a transcription factor to bind DNA if it is located within its DNA-binding motif (1). PTP-1B is directly inactivated by ROS-induced reversible oxidation of its catalytic site, Cys215, and this has been proposed as a mechanism for EGF-mediated mitogenic signaling (28, 163). Recent work by Barrett et al. (28) suggests that although both O2- and H2O2 are capable of inactivating PTP-1B, O2- may be the more relevant species due to its higher specificity, activity, and reversibility. This study also suggested that the specific cysteine modification involves S-glutathionylation, which is readily reversed to the active sulfhydryl group by thioltransferases. Reversible S-glutathionylation also appears to form the basis for redox regulation of c-Jun DNA binding (137).

Fig. 2. Oxidative modification of proteins as a potential mechanism for ROS-mediated cell signaling. A: the sulfhydryl (-SH) group of cysteine residues in proteins may be modified by O2- (or H2O2) to form various oxidized derivatives. S-glutathionylation appears to confer reversibility to such modifications by the action of thioltransferases (28, 137). B: formation of intramolecular disulfide bridges can alter protein activity by changes in conformation. For the bacterial oxidative stress-responsive protein OxyR, this leads to its activation (53, 278). In the nucleus, however, the reduced state of critical cysteines in some transcription factors, facilitated by disulfide-reducing systems (such as thioredoxin) and redox factor-1 (Ref-1), appears to promote DNA binding and transactivation (112, 151, 234). C: intermolecular disulfide linkages can mediate protein dimerization. Such a mechanism for activation of a protein kinase (PK) may involve its own dimerization (94) or that of a regulatory protein (RP), which leads to its dissociation from an inactive complex (173, 250). D: H2O2- or peroxidase-catalyzed dityrosine formation can induce protein cross-linking (149, 263). E: transitional metal-containing proteins may be targets of site-directed, metal-catalyzed oxidation by ROS produced by certain mixed-function oxidases (MFO), which “target” them for ubiquitination and degradation by proteases (124, 276). This is a potential mechanism for ROS-mediated alterations in protein stability.
Schmid et al. (255) recently described a novel mechanism for “redox priming” based on the ability of phosphocreatine in combination with H$_2$O$_2$ to serve as an alternate phosphate donor for the autophosphorylation of the insulin receptor kinase. In this study, the priming effect of H$_2$O$_2$ appears to involve conversion of four cysteine residues into sulfenic acid, which produces a structural change in insulin receptor kinase that allows for phosphocreatine binding at a site distinct from that of ATP.

Two (or more) cysteine residues within the same protein may be oxidized to form an intramolecular disulfide bridge(s), thereby altering its conformation (Fig. 2B). This is a well-described mechanism by which the oxidant stress-responsive transcription factor OxyR is activated by H$_2$O$_2$ in bacteria (53, 278). Although definitive evidence for such a mechanism in mammalian cell signaling is lacking, there is some suggestion that this may be an important mechanism for redox regulation. Conformational modulation of the p53 transcription factor through oxidation-reduction of cysteines at or near the p53-DNA interface appears to regulate both its binding and transactivating potential (234).

Similarly, the reduced state of cysteines in certain other transcription factors, facilitated by the activity Ref-1 and TRX, appears to promote DNA binding (111, 151, 315). Formation of intramolecular disulfide linkages has also been shown to be important in regulating enzyme activity. Gopalakrishna et al. (93) showed that the activity of PKC can be regulated by selenocompounds by forming disulfide bonds between accessible redox-active cysteine residues present within the catalytic domain of PKC.

Intermolecular disulfide bonds formed by the oxidation of cysteine groups from two identical or different proteins lead to homodimerization or heterodimerization reactions (Fig. 2C). A study by Gotoh and Cooper (94) suggests that ASK-1 activation by TNF-α is mediated by ROS-induced homodimerization of ASK-1. Interestingly, TRX also regulates ASK-1 activation in response to TNF-α by oxidant-dependent dissociation of TRX from the bound (inactive) complex (173, 250). It is unclear if the structural alteration in TRX that allows for its dissociation involves a dimerization reaction (by intermolecular disulfide bonds), an intramolecular disulfide linkage or oxidative modification of single cysteine residues. Regardless, in this example of redox-regulated activation of ASK-1 by TNF-α, ROS appear to function at two distinct regulatory steps: an indirect effect to induce dissociation of a regulatory protein from the bound (inactive) kinase and a direct effect on the kinase to mediate its dimerization and/or activation. A regulatory function similar to that of TRX has also been demonstrated for another cysteine-rich protein, glutathione S-transferase π-isozyme (GSTp) (5). In this study, Adler et al. (5) demonstrated that monomeric GSTp inhibited JNK by directly binding to it, whereas ultraviolet irradiation or H$_2$O$_2$ treatment caused GSTp oligomerization and dissociation of the GSTp-JNK complex, resulting in JNK activation. It has also been recently shown that products of mature granulocytes undergoing an oxidative burst rapidly oxidize monomeric pGlu-Glu-Asp-Cys-Lys (pEEDCK), an endogenous stem cell inhibitory peptide, to its active dimer by forming a disulfide linkage between cysteine residues (224).

Tyrosine residues have also been shown to participate in dimerization, oligomerization, and even polymerization of proteins (Fig. 2D). Dityrosine-dependent cross-linking of extracellular matrix proteins catalyzed by H$_2$O$_2$ or ovoperoxidase forms a protective coat around the freshly fertilized egg of sea urchin (263). In mammals, a similar peroxidative reaction is catalyzed by H$_2$O$_2$ or thyroid peroxidase in the coupling of diiodotyrosine to synthesize thyroxine (148). We have observed the ability of endogenous H$_2$O$_2$ from TGF-β1-stimulated fibroblasts to mediate dimerization of i-tyro- osine, a reaction that requires peroxidase activity (Thannickal et al., unpublished observations). Recently, Lambeth (149) described a homologue of gp91phox Duox, which contains an NH$_2$-terminal peroxidase homology domain and is involved in the biogenesis of tyrosine cross-links in the cuticle of Caenorhabditis elegans.

Metal-iron clusters in metalloenzymes occur widely in biological systems (131). The iron-sulfur tetranuclear [4Fe-4S] core is the most common of these prosthetic groups and may be an important target of ROS, particularly O$_2^-\cdot$ (135). Iron regulatory proteins, which contain [4Fe-4S] clusters, are sensitive to oxidation, but this does not appear to directly regulate their binding to the iron-responsive elements of untranslated mRNA to alter protein translation (39, 124). Such proteins may, however, be targets of site-directed, metal-catalyzed oxidation by ROS (Fenton chemistry), which “marks” them for ubiquitination and degradation (Fig. 2E) (124, 276). Thus in this manner, ROS may be capable of regulating cell function by altering the stability of certain metal-containing proteins.

**CONCLUSION**

The concept that ROS have “purposeful” roles as “regulators” of cell function or as “signaling molecules” has gained significant recognition over the past several years from studies done in laboratories worldwide. The evidence supporting this concept is based largely on the following criteria: 1) growth factors and cytokines are capable of generating ROS in a number of different cell types, 2) antioxidants and inhibitors of ROS-generating enzymatic systems block specific growth factor- and/or cytokine-activated signaling events or physiological effects, and 3) exogenous addition of oxidants activates the same cytokine- and/or growth factor-mediated signaling pathway or produces the same physiological effects. However, investigators must recognize potential technical drawbacks when applying these criteria to establish cause-effect relationships between ROS and cell signaling/regulation. First, the measurement of ROS in biological systems can be misinterpreted if the redox chemistries of the detector molecules used in these assays are not carefully considered. Secondly, antioxidants and enzymatic inhibitors have multiple potential targets, and their effects may be due
to nonspecific actions rather than their "intended" actions to degrade or scavenge ROS and inhibit ROS-generating enzymes, respectively. Finally, exogenous addition of oxidants as a "bolus" and/or in relatively high concentrations does not simulate the conditions of tightly regulated and "compartmentalized" ROS production in response to physiological ligands.

The identity of the specific oxidase(s) activated by growth factors and/or cytokines in nonphagocytic cells is still in need of clarification. Although there are numerous reports of the expression of protein subunits of the multicomponent phagocytic NADPH oxidase in such cells, it is not known if assembly or activation of the same or a similar enzymatic complex is responsible for ligand-stimulated ROS production. Recent cloning and characterization of the homologues of the phagocytic NADPH oxidase, the Nox (formerly Mox) family of oxidases (279), may lead to improved understanding of oxidases in cell signaling. Although PDGF has been shown to increase mRNA expression of Nox-1 in vascular smooth muscle cells (279), its role in the "early and immediate" generation of ROS after ligand binding is still unclear. A study by Thannickal et al. (291) on the ROS-generating effects of various ligands in lung fibroblasts suggest that the enzymatic sources and their regulation by mitogenic growth factors versus TGF-β1 are distinctly different. Taken together, it appears that a family of NAD(P)H-dependent oxidoreductases in nonphagocytic cells functions as generators of "redox signals" at the plasma membrane in response to growth factor and/or cytokine stimulation.

A major gap in our understanding of ROS signaling is the mechanism(s) by which these molecules transduce their cellular signals. The emerging evidence for the oxidative modification of proteins by ROS provides one of the more plausible mechanisms for such signaling. However, several questions remain. How is the specificity of action achieved? How are ROS "disposed" of after they have mediated their action? What is the mechanism for the "reversibility" of action or the cyclical "on-off" switch that would be expected (much like phosphorylation-dephosphorylation)? Recent work has begun to shed light on some of these questions. Barrett et al. (28) postulated that the specificity of $O_2^-$ (over the neutral $H_2O_2$) for PTP-1B inactivation may be related to the presence of positively charged residues surrounding the site of action. Similarly, the extreme sensitivity of OxyR to low concentrations of $H_2O_2$ appears to be related to conserved amino acid residues surrounding the two active-site cysteines (325). In addition, the site of ROS production (compartimentalization), the specific reactive species (O$_2^-$ versus $H_2O_2$) produced, and the concentration and kinetics of ROS generation are all likely to be important factors in determining the physiological actions and effects of ROS in cell signaling.

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