Reactive Oxygen Species: A Breath of Life or Death?

John P. Fruehauf and Frank L. Meyskens, Jr.

Abstract New insights into cancer cell–specific biological pathways are urgently needed to promote development of rationally targeted therapeutics. Reactive oxygen species (ROS) and their role in cancer cell response to growth factor signaling and hypoxia are emerging as verdant areas of exploration on the road to discovering cancer's Achilles heel. One of the distinguishing and near-universal hallmarks of cancer growth is hypoxia. Unregulated cellular proliferation leads to formation of cellular masses that extend beyond the resting vasculature, resulting in oxygen and nutrient deprivation. The resulting hypoxia triggers a number of critical adaptations that enable cancer cell survival, including apoptosis suppression, altered glucose metabolism, and an angiogenic phenotype. Ironically, recent investigations suggest that oxygen depletion stimulates mitochondria to elaborate increased ROS, with subsequent activation of signaling pathways, such as hypoxia inducible factor 1α, that promote cancer cell survival and tumor growth. Because mitochondria are key organelles involved in chemotherapy-induced apoptosis induction, the relationship between mitochondria, ROS signaling, and activation of survival pathways under hypoxic conditions has been the subject of increased study. Insights into mechanisms involved in ROS signaling may offer novel avenues to facilitate discovery of cancer-specific therapies. Preclinical and clinical evaluation of agents that modify ROS signaling in cancer offers a novel avenue for intervention. This review will cover recent work in ROS-mediated signaling in cancer cells and its potential as a target for developmental therapeutics.

What Are Reactive Oxygen Species and the Redox Balance?

Reactive oxygen species are emerging as critical signaling molecules (1–8). The term reactive oxygen species (ROS) encompasses a wide range of molecules. Free radicals are chemical species containing one or more unpaired electrons. Examples include the hydrogen atom, with one unpaired electron, most transition metal ions, nitric oxide, and oxygen, which has two unpaired electrons (3). The unpaired electrons of oxygen react to form partially reduced highly reactive species that are classified as ROS, including superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical, and peroxynitrite. Various enzyme systems produce ROS, including the mitochondrial electron transport chain, cytochrome P450, lipoxygenase, cyclooxygenase, the NADPH oxidase complex, xanthine oxidase, and peroxisomes (6). Mitochondrial oxygen metabolism is the dominant source of O_2^- that results from incomplete coupling of electrons and H^+ with oxygen in the electron transport chain. Under normoxic conditions, ROS are maintained within narrow boundaries by scavenging systems, as would be expected where fluxes of such species are involved in cell signaling (8, 9). Redox balance, the ratio between oxidizing and reducing species within the cell, plays a significant role in the regulation of signaling pathways, including kinase and phosphatase activity and gene expression through modulation of transcription factor function (10–12). Redox balance is achieved by various enzyme systems that neutralize toxic oxidants, such as ROS. Superoxide dismutases (SOD) catalyze the conversion of O_2^- to H_2O_2, which can then be converted to water by catalase or glutathione (GSH) peroxidase coupled with glutathione reductase. Other relevant scavengers include thioredoxin coupled with thioredoxin reductase, and glutaredoxin, which uses GSH as a substrate. GSH plays a central role in maintaining redox homeostasis, and the GSH to oxidized glutathione ratio provides an estimate of cellular redox buffering capacity (13).

How Do ROS Play a Role in Transformation and Signal Transduction?

ROS-mediated DNA damage has long been thought to play a role in carcinogenesis initiation and malignant transformation (Fig. 1A; ref. 14). Hydroxyl radicals, for example, react with pyrimidines, purines, and chromatin protein, resulting in base modifications, genomic instability, and alterations in gene expression. Mitochondrial DNA is a particularly vulnerable target because of its proximity to the electron transport chain constituents. ROS-mediated mutations in mitochondrial DNA have recently emerged as an important variable in carcinogenesis (15). Pathologic sources of transforming ROS include chronic inflammation secondary to infections or chronic chemical irritants (tobacco smoke, asbestos; refs. 16, 17). Transformed cells commonly lack cell cycle checkpoints and

Authors' Affiliation: Departments of Medicine and Biological Chemistry, Chao Family Comprehensive Cancer Center, University of California, Irvine, Orange, California

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Requests for reprints: John P. Fruehauf, Hematology/Oncology, University of California, Irvine, Cancer Center, Building 55, Room 321, 101 City Drive Cancer Center, Orange, CA 92668. Phone: 714-456-6310; Fax: 714-456-7668; E-mail: jfruehau@uci.edu.

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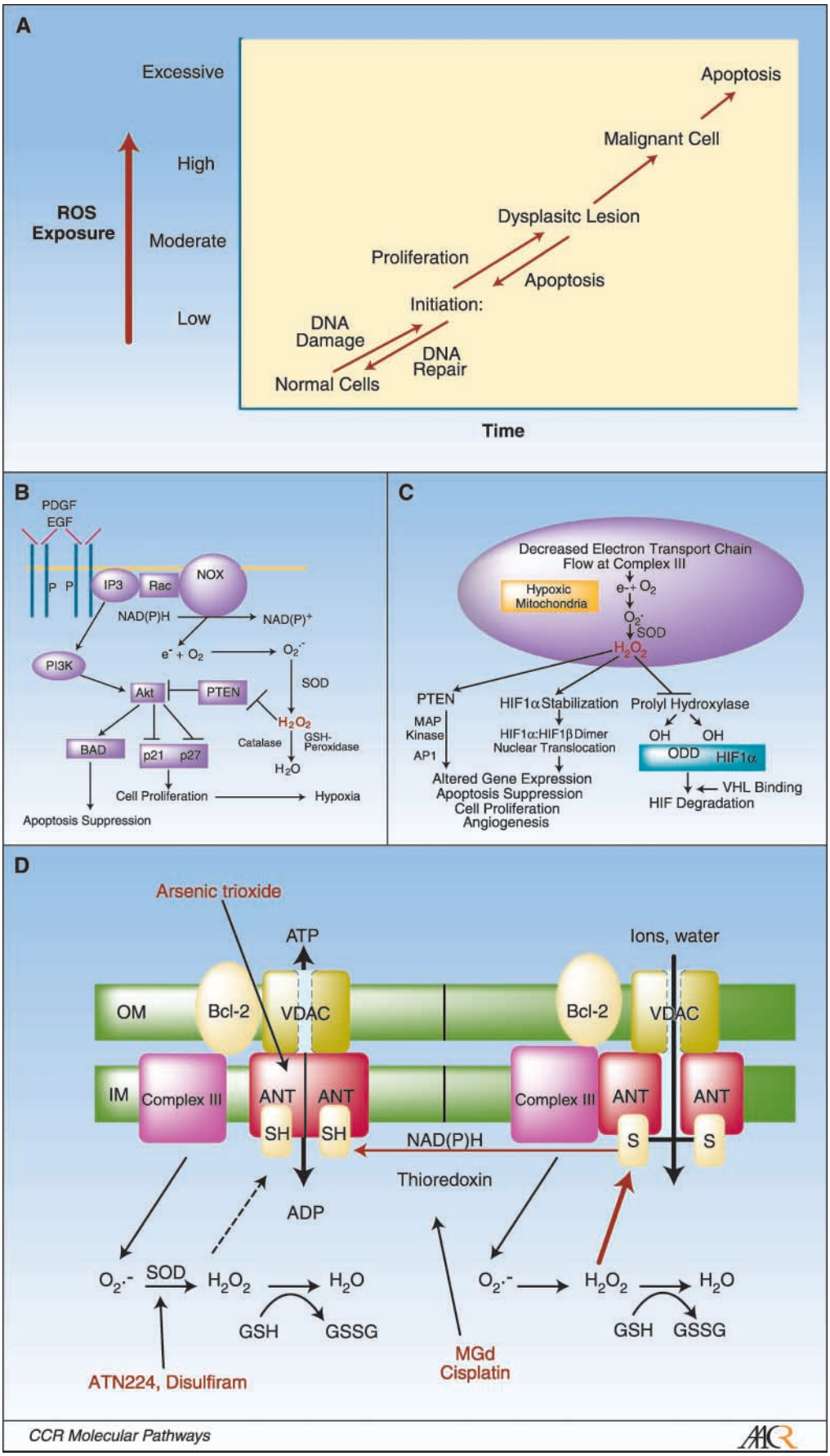

Fig. 1. *A,* chronic ROS exposure is carcinogenic. Excess levels are toxic to cancer cells. *B,* tyrosine kinase receptor signaling is amplified by ROS via inhibition of PTEN, stimulating cell proliferation and suppressing apoptosis. IP3, inositol 1,4,5-trisphosphate. EGF, epidermal growth factor. *C,* hypoxic mitochondria signal via superoxide and hydrogen peroxide to stabilize HIF1α and activate mitogen-activated protein (*MAP*) kinase signaling, promoting cell proliferation, apoptosis suppression, and angiogenesis. AP1, activator protein-1; ODD, oxygen-dependent degradation domain. VHL, von Hippel-Lindau. *D,* targeting ROS-sensitive components of the mitochondrial permeability pore offers a new avenue for therapeutic intervention. OM, outer membrane; IM, inner membrane; GSSG, oxidized glutathione. Fig. 1D adapted with permission from Armstrong (59).

overexpress oncogene growth factors and their tyrosine kinase receptors that drive cell proliferation, ultimately leading to tumor formation and chronic hypoxia (18). Several tyrosine kinase receptors have been shown to signal via ROS-dependent mechanisms (19, 20). Both the epidermal growth factor receptor and platelet-derived growth factor receptor signal in part through H_2O_2 generation (Fig. 1B). Ligand-induced receptor dimerization activates phosphatidylinositol 3-kinase, resulting in inositol 1,4,5-triphosphate activation of Rac, which, in turn, activates the NADPH oxidase complex to produce superoxide and downstream signaling through O_2^- and H_2O_2. H_2O_2 modulates signal transduction through its oxidation of the catalytic cysteine of protein tyrosine phosphatases, such as PTEN, preventing inactivation of tyrosine kinase signaling through activator protein-1 and Akt (21–23). H_2O_2-mediated inhibition of protein phosphatases contributes to both cellular proliferation and apoptosis suppression and links oncogene overexpression, a hallmark of many cancers, with ROS-mediated signaling (24). Oncogene growth factor activation and signal transduction drives cell proliferation beyond the carrying capacity of the resting vasculature. As few as 300 malignant cells are adequate for the production of a hypoxic environment that turns on angiogenesis (25). It is not surprising then that Akt activation by ROS can also support tumor cell survival under hypoxic conditions by increasing the translation of hypoxia inducible factor 1α (HIF1α; ref. 26).

What Is the Role of HIF Stabilization in Hypoxia?

Tumor survival in a hypoxic environment requires a coordinated adaptive response. Identification of mechanisms of oxygen sensing and its effect on cellular adaptations to hypoxia has been a critical task facing tumor biologists (27, 28). Initial studies suggested that HIF1α was a central regulator of hypoxic response (29–32). More that 70 genes are under its transcriptional control to facilitate survival under low oxygen pressures (29). HIF1α is constitutively expressed, but its half life is extremely short due to rapid hydroxylation by dioxygen, oxaloglutarate, and iron-dependent prolyl 4-hydroxylases (PHD 1, 2, and 3), located in the nucleus, cytoplasm, or both, respectively. After PHD-mediated hydroxylation of Pro^{564} and Pro^{402} in its oxygen-dependent degradation domain, HIF1α complexes with the β-domain of von Hippel-Lindau tumor suppressor protein, a recognition component of an E3 ubiquitin-protein ligase complex, and undergoes rapid NH_2- and COOH-terminal ubiquitination and proteolysosomal degradation (30, 31). Under normoxic conditions, the half-life of HIF1α is <5 min (31). Based on the oxygen requirements for PHD-mediated hydroxylation, it was initially postulated that this was the key oxygen sensor protein (27). However, inhibition of PHD does not occur until oxygen levels decrease below 5%, with maximal inhibition not seen until near-complete anoxia (32). Recent studies suggest that various oxygen species can promote HIF1α stabilization by inhibiting PHD activity, including nitric oxide and ROS, some of which may be of mitochondrial origin (33–35).

What Is the Role of Mitochondrial H_2O_2 in HIF Stabilization?

Based on the central role of oxygen in oxidative phosphorylation, it is not surprising that mitochondria can signal a cellular response when oxygen levels decrease (Fig. 1C; refs. 34, 35). Under hypoxic conditions, mitochondria participate in a ROS burst generated at complex III of the electron transport chain (36). When the partial pressure of oxygen is reduced, mitochondrial electron transfer from ubiquinol to cytochrome c_1 by the Reiske iron-sulfur protein is delayed, allowing electrons to bind to molecular oxygen, forming O_2^- (36). Superoxide is then converted to H_2O_2 by SOD (Mn-SOD in the mitochondrial matrix and Cu,Zn-SOD in the mitochondrial intermembrane space and cytosol). The resulting H_2O_2 efflux into the cytosol exerts an inhibitory effect on PHD activity, allowing HIF1α to accumulate, dimerize with HIF1β, and translocate into the nucleus where it modulates the expression of genes that favor survival under hypoxic conditions (Fig. 1C; ref. 29). Support for the role of mitochondrial ROS in HIF1α stabilization comes from work which shows that HIF1α stabilization can be blocked under hypoxic conditions if ROS production is abrogated in mitochondria that lack cytochrome *c* or that have been treated with small interfering RNA to knock down the Rieske protein (37, 38). However, HIF1α stabilization under anoxic conditions is independent on mitochondrial ROS (36). Although the mechanism whereby H_2O_2 inhibits PHD activity has yet to be elucidated, current efforts are focused on PHD iron oxidation (39).

Can Modulation of ROS Be Therapeutic?

ROS are increased in malignant cells in part as a result of oncogene signaling via the NADPH oxidase complex and by hypoxia-related mitochondrial ROS. Increased oxidant levels contribute to enhanced cell proliferation and apoptosis suppression (Fig. 1B and C). Two independent therapeutic strategies targeting these pathways are possible. One point of attack would be to increase ROS scavenging, thereby dampening H_2O_2 signaling and depressing tumor growth. An opposite approach would be to treat cells with agents that interfere with ROS scavenging, resulting in excess ROS that would trigger apoptosis (Fig. 1D; refs. 9, 40–42). Evidence to support a strategy to enhance scavenging is provided by studies showing that overexpression of SOD, glutathione peroxidase, or catalase decreased tumor growth *in vitro* and *in vivo* in mouse models (43–46). Although there are no specific agents available that selectively induce these enzyme systems, nutriceutical preparations are under study that show some promise (47). In opposition to increased scavenging are therapeutic maneuvers that interfere with ROS removal, leading to an accumulation of excess ROS. High levels of ROS can cause apoptosis by triggering mitochondrial permeability transition pore opening and release of proapoptotic factors (Fig. 1D; ref. 48).

How Does the Mitochondria Control Apoptosis?

The mitochondrial permeability transition pore complex is a highly regulated multimeric channel consisting of an inner membrane segment, the adenine nucleotide translocase (ANT), which imports ADP and exports ATP, cyclophilin D, intermembrane creatinine kinase, and the outer membrane voltage dependent ion channel (VDAC, porin). Chemotherapy agents modulate pore opening primarily by triggering DNA damage response pathways at cell cycle checkpoints (18). DNA repair pathways are coupled with apoptosis effectors to ensure that

irreparable damage will not be passed down to daughter cells. Drug-induced apoptosis results when cytosolic concentrations of pore opening proteins, such as Bax and Bak, increase above a critical threshold and are targeted to destabilize VDAC by chaperones such as Bid and Bim. VDAC destabilization increases ROS generation and promotes ion influx and ultimate mitochondrial membrane rupture, causing the release of the proapototic protein group, including cytochrome *c*, apoptosis-inducing factor, Smac/Diablo, procaspases, and Endo G (49, 50). On the other hand, hexokinases I and II (up-regulated by HIF), Bcl-2, Bid, and BCL-X_L (up-regulated by tyrosine kinase receptor and ROS signaling) exert antiapoptotic effects by stabilizing VDAC configuration (51, 52).

Are Mitochondrial Permeability Pores and Apoptosis Regulated by ROS?

In addition to attack by pore-destabilizing proteins, VDAC, which may regulate O_2^- flux from the mitochondria to the cytosol, is susceptible to superoxide-mediated mitochondrial permeability transition pore opening (53–55). Thus, VDAC can be a target of ROS buildup to stimulate apoptosis. The inner mitochondrial protein, ANT, is also a target of ROS modulation by virtue of its redox-sensitive cysteines, providing an additional mechanism by which drug-induced GSH depletion and loss of ROS scavenging may cause apoptosis (Fig. 1D; ref. 56). ANT contains three reduced cysteine residues in the 57, 160, and 257 positions. Oxidation-induced disulfide cross-linking of Cys^{160} with Cys^{257} results in mitochondrial permeability transition pore complex opening (57). Cross-linking of these Cys residues alters ANT conformation, inhibiting its ability to bind nucleotides and allowing calcium entry. Increased calcium is postulated to promote a cyclophilin D–ANT complex to form, which induces pore opening, leading to apoptosis (58). Glutathione prevents this cross-linking, whereas oxidized glutathione may mediate disulfide cross-link formation between Cys^{160} with Cys^{257}, resulting in apoptosis (59, 60). This disulfide can be reduced by thioredoxin coupled to thioredoxin reductase or by GSH coupled to glutathione reductase, reversing pore opening (61). ROS scavenging in the mitochondria is therefore required to promote a redox balance that maintains ANT in an active form that binds adenine nucleotides at both high- and low-affinity sites, preventing calcium from reaching cyclophilin D, thereby preventing pore opening.

What Drugs Are Available to Inhibit ROS Scavenging?

Therapeutic strategies that promote ROS accumulation and apoptosis have been explored based on the availability of drugs that interfere with scavenging (Fig. 1D; refs. 42, 62, 63). Agents that deplete GSH, such as buthionine sulfoximine and arsenic trioxide, have shown *in vitro* and clinical activity (56, 64–67). Arsenic trioxide may act directly on VDAC to induce pore opening (68). Inhibition of Cu,Zn-SOD by agents that chelate Cu, such as disulfiram and ATN224, have shown *in vitro* and *in vivo* clinical activity (69–72). Both buthionine sulfoximine and disulfiram were found by our group to be active against melanoma *in vitro* (65, 69). Melanoma cells are postulated to contain excess levels of ROS secondary to dysregulated melanin synthesis (42, 65). Under oxidizing conditions, melanin is converted from an antioxidant to a prooxidant macromolecule (73).

Inhibition of thioredoxin, which maintains ANT in a reduced state, is another potential target for disruption of ROS scavenging (74). Flavanols, such as quercetin, are capable of causing cancer cell death via inhibition of thioredoxin, and their activity is enhanced by superoxide anions (75). A new compound, motexafin gadolinium, which was initially developed as a radiosensitizer, is an effective inhibitor of thioredoxin, and is currently undergoing phase III clinical trials (76, 77). Motexafin gadolinium is relatively tumor specific based on its porphyrin-like structure that is preferentially taken up by cancer cells. It induces oxidative stress by a mechanism of futile redox cycling (due to transfer of electrons from reduced substrates to O_2^- to produce ROS). A wide spectrum of critical reducing proteins, including GSH and reduced thioredoxin, are oxidized by motexafin gadolinium. Motexafin gadolinium not only inhibits thioredoxin but also converts this scavenger to an ROS generator, which further contributes to apoptosis induction (78). Of note, conventional chemotherapy agents can inhibit thioredoxin, including melphalan, carmustine, cisplatin, and oxaliplatin (79). Thus, combining these agents in synergistic combinations may prove to be the most useful approach. To date, the toxicity of these newer ROS-targeted agents has been tolerable.

In summary, ROS species are involved in carcinogenesis, promotion of transformed cell growth, stabilization of HIF1α to promote angiogenesis, and regulation of mitochondrial apoptotic programs. Scavenging of H_2O_2 in transformed cells can effectively diminish tumor growth by blocking growth factor receptor signaling and by preventing peroxide-mediated stabilization of HIF1α. Although inhibition of redox signaling through enhanced ROS scavenging has been attempted as a chemoprevention strategy early in the transformation process, few studies have successfully showed proof of this principle for patients with advanced disease (80). It is unlikely that abrogation of ROS signaling can significantly affect patient outcomes due to the complexity of redundant pathways supporting cancer growth (18). On the other hand, enhancing mitochondrial ROS production to trigger apoptosis presents an attractive target because this organelle controls cellular decisions to live or die. Cancer-specific therapies may ultimately benefit from the increased ROS produced by hypoxic mitochondria. Through the inhibition of ROS scavenging, increased levels of ROS can be seen as the Achilles' heel of cancer cell metabolism. The next decade should reveal the truth or consequences of this approach.

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