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COMPREHENSIVE INVITED REVIEW

Redox Control of the Survival of Healthy and Diseased Cells

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Abstract

Cellular redox homeostasis is the first line of defense against diverse stimuli and is crucial for various biological processes. Reactive oxygen species (ROS), byproducts of numerous cellular events, may serve in turn as signaling molecules to regulate cellular processes such as proliferation, differentiation, and apoptosis. However, when overproduced ROS fail to be scavenged by the antioxidant system, they may damage cellular components, giving rise to senescent, degenerative, or fatal lesions in cells. Accordingly, this review not only covers general mechanisms of ROS production under different conditions, but also focuses on various types of ROS-involved diseases, including atherosclerosis, ischemia/reperfusion injury, diabetes mellitus, neurodegenerative diseases, and cancer. In addition, potentially therapeutic agents and approaches are reviewed in a relatively comprehensive manner. However, due to the complexity of ROS and their cellular impacts, we believe that the goal to design more effective approaches or agents may require a better understanding of mechanisms of ROS production, particularly their multifaceted impacts in disease at biochemical, molecular, genetic, and epigenetic levels. Thus, it requires additional tools of omics in systems biology to achieve such a goal. *Antioxid. Redox Signal*. 15, 2867–2908.

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I. Introduction

In 1954, Gerschman *et al.* first stated that oxygen (O_2) toxicity is due to the incompletely reduced form of O_2 (103). The subsequent discovery of the relationship between free radicals and aging by Harman in 1956 initiated research on free radicals in biological systems (125). Another important event in the free radical field was the discovery of superoxide dismutase (SOD) by McCord and Fridovich in 1969, which impelled the free radical research widely focused on biological areas (240).

Reactive oxygen species (ROS) is a collective term that describes O_2-derived free radicals, mainly including superoxide anion ($O_2^{\bullet -}$), hydroxyl radical (HO•), nonradical species hydrogen peroxide (H_2O_2), and a variety of their reaction products.

In physiological processes, ROS can regulate many signaling pathways important for cell growth, proliferation, survival, and motility, as typically represented by the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/Akt pathways. Also, ROS production appears to be crucial for the regulation of innate immune responses toward invading microbes. In general, production of ROS is less harmful to cells, as antioxidant systems exist intracellularly that protect cells from oxidative damage. However, when accumulation of ROS occurs, poisonous properties of ROS prevail and damage fundamental cell constituents, including DNA, proteins, and lipids. Over the years, ROS have been demonstrated to participate in many pathological events. In the following section, we will first give a brief description of the source of ROS and intracellular defensive systems; in the second part, we will describe the role of ROS in cell survival and death under normal physiological conditions; the third part is mainly focused on the role of ROS during pathological processes, including atherosclerosis, ischemia and reperfusion injury, neurodegenerative disorders, diabetes mellitus (DM), and carcinogenesis. Finally, we will provide an overview on some cases of the translational application in ROS-targeting treatments.

II. Source of ROS and Influential Elements

A. ROS production

Generation of ROS at different sites in cells is thought to be an inevitable event that contributes to redox signal trans-

duction from the organelle to cytosol and nucleus under normal physiologic conditions. When excess oxidants are produced, oxidative stress occurs and triggers cell death or pathological oxidative damage.

Mitochondria are thought to be the major intracellular source of ROS in most mammalian cells. During the respiration, an estimated 1%–2% of O_2 gains an electron and is reduced to superoxide anion ($O_2^{\bullet -}$) (41), which can be converted to H_2O_2 (35). This percentage may be even lower (0.15%) according to a recent study (346). Superoxide anion ($O_2^{\bullet -}$) is produced by the one-electron reduction of O_2 and is the proximal form of ROS generated in mitochondria. Superoxide anion ($O_2^{\bullet -}$) can be converted to H_2O_2 and the highly reactive hydroxyl radical (HO•). Compared to superoxide anion ($O_2^{\bullet -}$), H_2O_2 is much more stable and can diffuse through biological membranes with the potential to act as a long-range signaling molecule (26, 207).

The respiratory chain is localized in the inner membrane of the mitochondrion and is thought to be the major source of superoxide anion ($O_2^{\bullet -}$) production (210). Among components of the respiratory chain, complex I and III are found to be the main sites of superoxide anion ($O_2^{\bullet -}$) production (83, 183, 210, 254) (Fig. 1). Two possible mechanisms are involved in ROS production in complex I. One is *via* reverse electron transfer (RET), by which electrons enter into complex I through CoQ-binding sites, and this process is abolished in the presence of rotenone (12, 191, 254). The other is rotenone-induced ROS production that requires a very high degree of reduction of redox carriers upstream of the rotenone binding sites in complex I (12, 188). Complex III is another important source of ROS generated by mitochondria (83, 183, 372). When supplied with $CoQH_2$ and the Q_i site is inhibited by antimycin, complex III produces large amounts of superoxide anion ($O_2^{\bullet -}$) from the reaction of O_2 with an ubisemiquinone bound to the Q_o site (372, 420). In addition, some key redox enzymes such as cytochrome b5 reductase, monoamine oxidases, and dihydroorotate dehydrogenase, at sites in the outer membrane and the sites in the matrix interacting with the matrix NADH pool, and the CoQ pool within the inner membrane, also contribute to the production of ROS in mitochondria (42, 183).

In addition to mitochondrial sources, ROS can also be produced by other pathways. For example, accumulating evidence suggests that protein folding and generation of ROS as byproducts of protein oxidation in the endoplasmic reticulum (ER) are closely linked events (226). ROS generated by oxidative protein folding in ER have been estimated to correspond to ~25% of total ROS generated in a cell (370). Protein disulfide isomerase (PDI) and ER oxidoreductin 1 (ERO1) are two major enzymes responsible for oxidative protein folding in ER. As an important enzyme in promoting protein folding in ER, PDI has multifunctional roles in catalyzing disulfide bond formation, isomerization, and reduction. In the process of oxidative protein folding, PDI receives electrons through catalyzing disulfide bond formation, and is converted to the reduced form, which then transfers electrons to ERO1 to recycle itself (226). ROS are generated when the molecular O_2 accepts the electrons from ERO1 based on flavin-dependent redox chemistry (Fig. 1). However, uncontrolled oxidation of disulfide bonds will increase the oxidant level of ER, resulting in protein misfolding and even oxidative stress. Thus, under normal conditions, the function of PDI/ERO1 is properly regulated according to the redox state in ER to minimize the intrinsic toxicity caused by ROS. For example, the activity of ERO1 can be regulated through the free flavin adenine nucleotide (FAD) available for ERO1 according to the cell metabolic state (369). ROS can also be induced in the ER through an indirect mode in which Ca^{2+} leakage into the cytosol

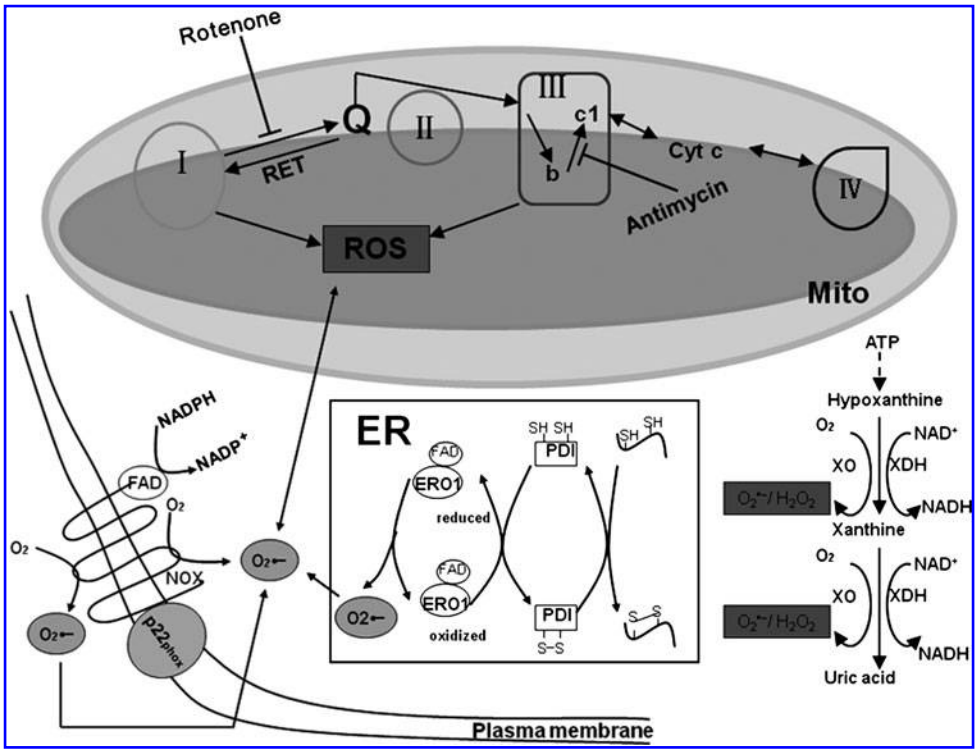

FIG. 1. Overview of reactive oxygen species (ROS) production. Mitochondria are the major producers of ROS as byproducts of electron flow in the respiratory chain, principally from complex I and III. In addition, endoplasmic reticulum (ER)-derived, NADPH oxidase (NOX)-derived, and xanthine oxidase (XO)-derived ROS also contribute to the generation of intracellular ROS.

triggers the production of ROS in mitochondria (64, 226). Alternatively, as the protein folding process is energy dependent, emergence of a large number of unfolded and misfolded proteins can cause depletion of adenosine triphosphate (ATP), which stimulates the production of ATP in mitochondria, consequently increasing ROS production (226).

NADPH oxidases (NOXs) comprise a family of ROS-producing enzymes, including seven members (NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2), each with the different tissue distribution and cell-type-specific subcellular localization (Table 1) (23, 192). Among these, NOX2 was the first discovered, and its biochemical features have been best studied. NOX2 is present both in the intracellular compartment and at the plasma membrane in close association with the transmembrane subunit $p22^{phox}$. NOX2-derived ROS are important to phagocytes, including neutrophils and macrophages, in exerting their host defense by killing invading microbes in the inflammation response (23, 192). The following studies indicate that NOX2 has a wide distribution in many tissues, and exerts an important role in physiological events; further details about the physiological roles of NOXs will be provided in section III.D. Since the discovery of ROS induced by NOXs in nonphagocytes, increasing numbers of scientific reports have shown that NOXs are predominantly involved in a variety of pathogenic processes, such as ischemia-reperfusion, diabetes, neurodegenerative diseases, and atherosclerosis, especially in the endothelial dysfunction (23, 48, 285, 305). The basic catalytic subunit of NOXs contains a C-terminal dehydrogenase domain featuring a binding site for NADPH and a bound FAD, as well as an N-terminal domain consisting of six transmembrane alpha helices that bind two heme groups (23, 212, 285). Once activated, cytosolic NADPH transfers its electrons to FAD, which in turn passes electrons sequentially to the two hemes and ultimately to molecular O_2, forming superoxide anion ($O_2^{\bullet -}$) (212, 285) (Fig. 1).

Mammalian xanthine oxidase (XO) and xanthine dehydrogenase (XDH) are interconvertible forms of the same gene product, known as xanthine oxidoreductase (XOR). XOR is a cytosolic enzyme. Under normal circumstances, most XOR exists in the form of NAD-dependent cytosolic dehydrogenase (XDH). During limited proteolysis and oxidation of sulfhydryl (SH) groups, XDH can be irreversibly converted to XO (9). XO and XDH exhibit a broad specificity toward reducing substrates. XOR catalyzes oxidation of hypoxanthine and xanthine, which are the metabolic products of adenine nucleotides, to produce uric acid as the final product in the presence of molecular O_2 (128). Superoxide anion ($O_2^{\bullet -}$) is a byproduct during this metabolic process (Fig. 1). Both enzymes can reduce molecular O_2 to superoxide anion ($O_2^{\bullet -}$) and H_2O_2, although XDH is characterized by high reactivity toward NAD^+, but low reactivity toward O_2, whereas XO has a high reactivity toward O_2, but negligible reactivity toward NAD^+ (134). Under normal conditions, a large amount of NAD^+ is generated to inhibit the production of ROS by XDH (264).

UV and ionizing radiations have been demonstrated to harm genomic DNA, amino acids, and other important cellular molecules either directly or indirectly by generating large amounts of oxidants that will trigger oxidative stress (251). Metals, due to their capacity to lose electrons, are thought to be primarily toxic by virtue of their potential to generate ROS. Thus, exposure to high concentrations of a single heavy metal can result in ROS accumulation and potentially, oxidative damage (377). Members of the cytochrome

Table 1. Summary of the Tissue Distributions, Subcellular Locations, and Main Functions of NADPH Oxidases Enzymes

	Tissue distribution	*Subcellular localization*	*Main function of Nox-derived ROS*
NOX1	Colon epithelia, uterus, smooth muscle, prostate, retinal pericytes, neurons, placenta, osteoclastes, astrocytes, microglia	Plasma membrane, nucleus, endosome	Initiation of necrosis or hypertrophy, promotion of cell growth and migration
NOX2	Phagocytes, VSMCs, endothelium, fibroblasts, skeletal muscle, CNS, endothelium, hepatocyte, cardiomyocytes	Plasma membrane, endosome membrane and phagosome	Host defense, promotion of necrosis, NF-κB activation and angiogenesis
NOX3	Inner ear, lung endothelial cell, fetal spleen, fetal skull, fetal kidney, brain	Plasma membrane	Association with TRPV1 channel
NOX4	Kidney, melanoma cells, smooth muscle cells, endothelial cells, neurons, fibroblasts, osteoclasts, keratinocytes, hepatocytes	Focal adhesion, nucleus, endoplasmic reticulum	Promotion of cell differentiation, growth, survival, hypertrophy, migration
NOX5	Lymphatic tissue, testis, VSMCs, endothelial cells, spleen, uterus, prostate cancer cells	Internal membrane, plasma membrane	Activation of growth and proliferation, increase in the inflammatory gene expression
Duox1	Thyroid, airway epithelia, tongue epithelium, cerebellum, testis	Plasma membrane	Defense (sterilization) and inflammation
Duox2	Thyroid, airway epithelia, salivary and rectal glands, gastrointestinal epithelia, uterus, gall bladder, pancreatic islets	Plasma membrane	Thyroid hormone (T4) production defense(sterilization) and inflammation

CNS, central nervous system; NOX, NADPH oxidase; ROS, reactive oxygen species; TRPV1, transient receptor potential cation channel subfamily V member 1; VSMC, vascular smooth muscle cells.

P450 (CYP) family are associated with ROS generation caused by the metabolism of toxic compounds (417), and upregulation of CYP has been linked to ROS production (280). Accordingly, drugs that inhibit P450 activity can inhibit ROS production and protect cells from ROS-induced damage after ischemia, or block the formation of catechol estrogens and their subsequent oxidation, thus decreasing oxidative damage (111).

Other metabolic pathways such as the NOS and arachidonic acid pathways can also cause the generation of ROS in most cell types (28, 273).

B. Antioxidants

Under physiologic conditions, ROS accumulation is limited by numerous endogenous antioxidant defense systems, including both enzymatic and nonenzymatic antioxidant mechanisms that can either scavenge ROS or prevent their formation. Here we will provide a brief description of the actors that participate in the first line of defense in the detoxification process of products resulting from oxidative stress.

Enzymatic antioxidants include SODs family, glutathione peroxidases (GPxs), peroxiredoxins (Prxs), catalases (CATs), *etc*. (218). SODs comprise a family of metal-containing proteins that catalyze dismutation of superoxide anion ($O_2^{\bullet -}$) to form H_2O_2 and O_2 (12, 168, 218). In most eukaryotic cells, SODs exist in the form of a copper-zinc SOD isoform in the cytoplasm, nucleus, and plasma membrane, whereas the manganese SOD (MnSOD) isoform is primarily located in mitochondria. SODs play important roles in the detoxification of over-produced superoxide anions ($O_2^{\bullet -}$) and their mutations are correlated with a variety of diseases such as central nervous system (CNS) disorders (314). H_2O_2 produced by SODs is also harmful to cells, and is converted to the final product of water (H_2O) mainly by GPxs, CATs, or Prxs (168, 218) (Fig. 2). Glutathione peroxidase-1 (GPx-1), which exists in the cytoplasm and mitochondria of most cell types, inactivates peroxides by using glutathione as a source of reducing equivalents (218). It reduces lipid hydroperoxides to their corresponding alcohols and free H_2O_2 to H_2O. CATs are heme-containing enzymes that convert H_2O_2 to H_2O and O_2, and are largely localized in subcellular organelles such as peroxisomes (81, 218). Prxs are a family of thiol-dependent antioxidants that scavenge cytosolic or mitochondrial peroxides. Biochemical reactions of Prxs involve oxidation of peroxidatic cysteines of their catalytic sites to sulfenic acids (Cys-SOH), which in turn form disulfide bonds with another cysteine at the C-terminal subunit. Prxs are coupled with the thioredoxins (Trxs), which maintain the reductive form of Prxs (4, 81, 404) (Fig. 2). On the other hand, Trxs can catalyze the reversible reduction of protein disulfide bonds and their active-site cysteine is regenerated by Trx reductase and NADPH (61, 257). The antioxidant system composed by the enzymatic antioxidants is shown in Figure 2.

In addition to antioxidant enzymes, other nonenzymatic antioxidants such as L-γ-glutamyl-L-cysteinyl glycine (glutathione) (GSH), NAD(P)H, vitamin C, vitamin E, uric acid, and bilirubin play important roles in the scavenging of ROS. GSH is the most abundant cellular antioxidant, and prevents protein thiol groups from oxidation, either directly by reacting with reactive species or indirectly through GPxs (367). In addition, GSH participates in protecting protein cysteine

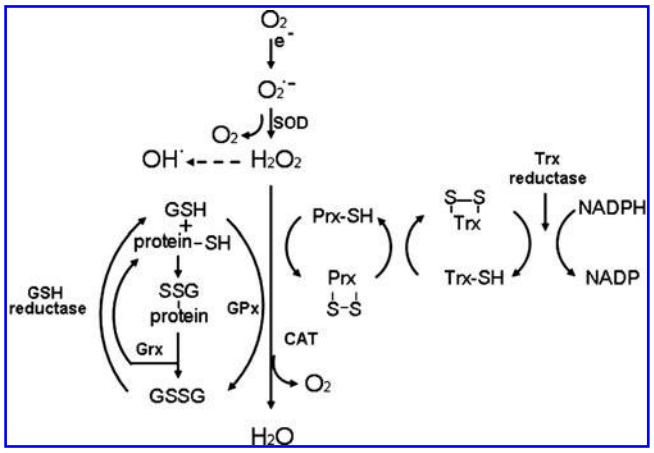

FIG. 2. Enzymatic antioxidant system. SODs catalyze superoxide ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2). H_2O_2 can be converted to the final product of water (H_2O) mainly by GPxs, CATs, or Prxs. The active state of Prxs is coupled with thioredoxins (Trxs) system, which supplies the reductive form of Prxs, and Trxs active-site cysteines are regenerated by Trx reductase and NADPH. In addition, L-γ-glutamyl-L-cysteinyl glycine (glutathione) (GSH) participates in the formation of protein-SSG, which is then reduced by glutaredoxins (Grxs) to produce protein-sulfhydryl (SH) and GSSG. GSSG, glutathione disulfide; SSG, glutathione mixed disulfide.

residues from oxidation by forming protein-glutathione mixed disulfide (protein-SSG) mixed disulfides, which can be reduced by the thioltransferase glutaredoxins (Grxs) to form protein-SH and glutathione disulfide (GSSG) (242). The reduction of GSSG to GSH is conducted by GSH reductase (Gr) (Fig. 2). Vitamin C, also known as ascorbic acid, protects cells against oxidative damage in the aqueous phase of the cytoplasm (132, 163), whereas vitamin E (tocopherol), a typical fat-soluble hydrophobic antioxidant, prevents lipid membrane peroxidation reactions by reacting with lipid radicals within the lipid environment (132, 163).

C. ROS targets

1. Lipids. The cellular membrane system is mainly composed of lipids and proteins, and phospholipids are predominant membrane components in eukaryotic cells. It is known that oxidants are more soluble in the fluid lipid bilayer than in the aqueous milieu, so the membrane phospholipids are more vulnerable to ROS, resulting in destruction of the membrane functions through altering the features of fluidity and permeability (229). Lipid peroxication (LPO) generates hydroperoxides and endoperoxides (262), which are degraded to the reactive carbonyl species (RCS), the secondary products of LPO. The diverse RCS can induce modifications or do damage to proteins and DNA bases. Aldehydes are also formed during LPO as secondary degradation products. They can react with proteins, DNA, and phospholipids to form covalent adducts. Among these, 4-hydroxynonenal (HNE), an aldehyde end product of lipid peroxidation, is the most studied and found to participate in many pathological processes. HNE is highly reactive and can react with DNA to cause carcinogenesis effects, or modify amino acids by the Michael addition reaction to alter their activity (60). Under normal physiological conditions, LPO products act as second

messengers in signaling pathway that regulate gene expression, whereas overproduction of these products is mutagenic and carcinogenic, and is relevant to many diseases, including cardiovascular diseases, neurological disorders, aging, and cancer (108, 295, 399).

2. Proteins. Proteins are also targeted by ROS through the oxidation of their thiol containing amino acids. Two thiols can be oxidized through formation of disulfide bonds, which are critical to protein structure and function. Thus, thiol containing proteins can act as redox switches, to sense concentrations of oxidative stressors and to take part in important regulatory and signaling pathways (106, 116). For example, protein tyrosine phosphatases (PTPs) contain cysteine residues in their catalytic domain, and are susceptible to oxidative inactivation. PTPs control the phosphorylated state of a series of signal-transduction proteins, and the redox-sensitivity of PTPs enhances the effect of ROS in cellular signaling pathways.

A paradigm of protein oxidation in the physiological setting is S-glutathionylation (102). Protein glutathionylation is a process of reversible post-translational protein modification including thiol-disulfide exchange, or by reaction of a reduced protein-SH or GSH with an oxidized SH derivative like sulfenic acid (102). S-glutathionylation of proteins plays an important role in protecting protein cysteines from irreversible oxidative damage. Protein glutathionylation can also change the protein activity and have a role in redox signaling. Under normal physiological conditions, the glutathionylation status of some proteins is important for many vital functions such as actin polymerization, transcription factor activation, and apoptosis (67, 147, 242, 309).

3. DNA. Free radicals generated by UV, biotoxins, and other environmental genotoxins can cause cellular DNA oxidative damage through formation of 7, 8-dihydro-8-oxoguanine (8oxodG) (121, 310). Compared to nuclear DNA, mitochondrial DNA (mtDNA) is more sensitive to oxidative stress-induced damage (310, 379), a circumstance that may be related to its open circular structure unprotected by histones, and its position in mitochondria as a main source of intracellular ROS (408). Mitochondria play a critical role in the initiation of apoptosis and in recent years, mtDNA mutations have been linked to various diseases (408). Thus, mtDNA may represent an important target for triggering cell death or diseases during oxidative stress-induced damage.

III. Role of ROS Under Normal Physiological Conditions and in Oxidative Stress

Under normal physiological conditions, ROS are inevitably generated as byproducts of oxidative phosphorylation (OXPHOS), the activation of neutrophils during inflammation and infection, and other physiological reactions. Indeed, ROS are currently thought to be important signaling messengers for cell proliferation, differentiation, apoptosis, and other critical events during development when the balance between oxidants and antioxidants is well controlled. The imbalance caused by ROS and concomitant antioxidant system is prone to induce oxidative stress. A slight imbalance by oxidative stress can be reversed to the original state of cells through self-control. However, more severe oxidative stress has destructive effects on cells, leading to cell dysfunction or cell death. In the following section, we give a brief introduction to the role of ROS as messengers in signaling cascades and in regulating redox-sensitive transcription factors under normal physiological conditions.

A. Role of ROS in signaling cascades

Under physiological conditions, ROS participate in the regulation of multiple cell functions in response to diverse stimuli from extracellular and intracellular sources. Extracellular cytokines such as platelet-derived growth factor (PDGF), epithelial growth factor (EGF), insulin, and tumor necrosis factor-α (TNF-α), stimulate plasma membrane receptors, which can induce ROS generation (54, 249). In addition, unfolded protein response (UPR)-induced ER-stress and diverse intracellular metabolic processes can also produce ROS as byproducts (226). Proteins with regulatory ability can be modified by ROS through reversible oxidation of thiols to mixed disulfides, vicinal dithiols to disulfides, zinc-fingers to disulfides, or methionines to methionine sulfoxides. Here we will review three selected redox signaling pathways important for cell survival.

1. PI3K/Akt signaling cascade. ROS have been implicated as regulators of cell survival and cell death by modulating multiple signaling cascades. Signaling through PI3K is pivotal to cell growth and survival. PI3K is typically activated following interaction of growth factors with receptor tyrosine kinases (RTK). It has been shown that EGF or PDGF activation of the PI3K pathway can result in ROS generation, mainly by NOXs, and the generated ROS can modulate some regulators of the PI3K signaling cascade to promote or suppress downstream signaling (184, 374) (Fig. 3). Phosphatase and tensin homolog (PTEN), a major antagonist of the PI3K/Akt signaling cascade, is susceptible to H_2O_2-dependent oxidative inactivation (206), as Cys^{124} in the active site of PTEN can specifically form a disulfide with Cys^{71} during reversible oxidation by H_2O_2. Inactivation of PTEN leads to the accumulation of phosphatidyl-inositol 3,4,5-triphosphate (PIP3) at the plasma membrane, resulting in activation of Akt and its downstream signaling (189). ROS can inactivate PTEN, in turn influencing the intracellular redox balance. Previous studies have indicated that PTEN deficiency in mouse embryonic fibroblasts (MEFs) correlated with deregulated expression of several antioxidant enzymes, including Prx1, 2, 5, and 6, and Cu and Zn SOD, resulting in an increase in basal ROS levels and susceptibility to oxidative injury (148). PTEN is a tumor suppressor, and PTEN dysfunction has been found in many malignant tumors (338). One possible explanation for this observation is that ROS levels in cancer cells are always upregulated and that the oxidized environment is unfavorable for PTEN activity.

Akt (also known as protein kinase B, PKB) is a serine/threonine protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, cell proliferation, and apoptosis, and has a pivotal role in PI3K/Akt signal cascades. Previous studies have indicated that Akt can also be directly regulated by oxidation of its cysteines to form an intramolecular disulfide bond between Cys^{297} and Cys^{311}, thus resulting in dephosphorylation by protein phospatase PP2A and inactivation (253). However, this is a reversible process.

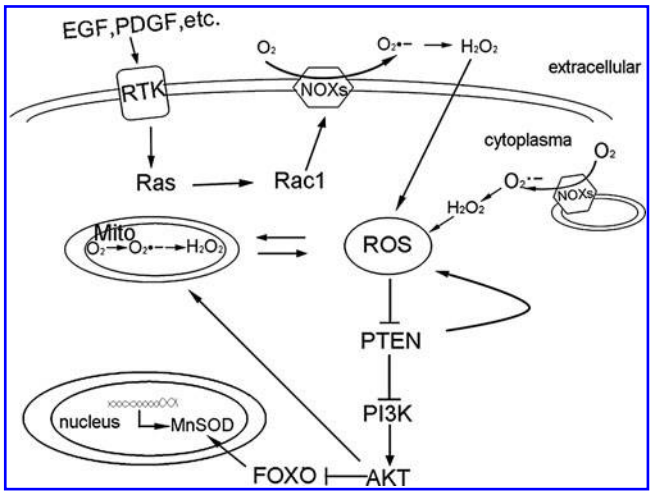

FIG. 3. Redox regulation of phosphoinositide 3-kinase (PI3K)/Akt signaling cascade. Extracellular stimulations by epithelial growth factor (EGF), platelet-derived growth factor (PDGF), and other growth factors can trigger the activation of NOXs through activated Ras and Rac1. NOX-generated ROS can depress the activity of PTEN, a negative regulator of PI3K/Akt signaling, resulting in activated Akt and the downstream signaling transduction such as inhibiting the transcriptional activity of Forkhead box-O (FoxOs) and their target genes with antioxidant effects such as manganese superoxide dismutase (*MnSOD*). Activated Akt contributes to the redox levels by increased oxygen (O_2) consumption, which may be a positive feedback for the activation. The inactivity of PTEN can also promote the upregulation of intracellular redox levels through some mechanisms such as some antioxidant genes downregulated.

When ROS are decreased, Akt can be reduced to its active form to exert its prosurvival role. In addition, the activity of Akt also has an effect on the intracellular redox status. It has also been shown that activation of Akt can sensitize cells to ROS-mediated apoptosis by increasing intracellular ROS through increased O_2 consumption and by inhibiting the expression of ROS scavengers downstream of Forkhead box-O (FoxO) (266).

2. MAPK signaling pathway. The MAPK cascade comprises a complex signaling network responsible for cellular events, such as cell proliferation, differentiation, and death. It includes MAP kinase kinase kinase (MAP3K), MAP kinase kinase (MAP2K), and MAPK. C-Jun N-terminal kinase (JNK), p38 MAPK, and extracellular signal-regulated kinase (ERK) are three well-characterized MAPKs. ERK is usually activated by growth factors and cytokines, and is involved in cell proliferation, whereas JNK and p38 signaling pathways are mainly activated by various stress stimulants, including oxidative stress. It has been indicated that the mild levels of ROS activate the growth factor receptor-related ERK pathway and have mitogenic activity (40). However, over-abundant ROS cause oxidative stress and activate the stress-activated protein kinases (SAPKs) JNK and p38. Many kinds of pro-oxidants such as H_2O_2, arsenite, and UV irradiation have been shown to activate JNK and p38 kinase, and knockdown of JNK and/or p38 kinase suppresses stress-induced apoptosis (363, 366). JNK has been determined to phosphorylate p66 isoform of ShcA adaptor protein (p66ShcA) at serine 36, which is critical for response to the oxidative stress-induced cell death (201). Mice lacking p66ShcA are less susceptible to oxidative stress and have an extended lifespan (243).

ASK1, as a MAP3K, can be activated by various types of stress, such as oxidative stress and ER stress, and thus induces apoptosis. Trxs are negative regulators of ASK1, and the reduced form of Trxs can interact with the N-terminal regulatory domain of ASK1 to form an inactive complex. After exposure to TNF-α or ROS-induced oxidation by other stress stimulations, Trxs dissociates from ASK1, resulting in the activation of ASK1 (142). JNK and p38 are two targets of ASK1, and activation of ASK1 triggers the activation of JNK, p38 kinase, and subsequent apoptosis signaling. Ample evidence indicate that ASK1-induced p38 activity by the endogenous oxidative stress is related to physiological aging (141, 143). The longevity of Snell dwarf mice showing a resistance to oxidative stress in their liver cells has been attributed to their higher abundance of the Trx-ASK1 complex, as compared to the aged dwarfs (141).

In addition to the proapoptotic effects, activation of JNK and p38 can also promote cell survival, which may be determined by the duration and extent of oxidative stress. As important members of the antioxidant system, Trxs oxidation may involve an extensive and prolonged oxidative stress that cannot be repaired. Thus, the oxidative stress-induced activation of ASK1 may be a switch for survival or death in the redox signaling effect in MAPK cascades (Fig. 4). This hypothesis was demonstrated in ASK1-deficient cells (236). There was no obvious difference between the ASK1 $-/-$ MEFs and the ASK1 $+/+$ MEFs in the transient activation of JNK and/or p38 by ROS, whereas the sustained activation of JNK- and/or p38-induced apoptosis by serious oxidative stress was significantly suppressed in the ASK1 $-/-$ cells (364).

3. Wnt-β-catenin signaling. Dishevelled (Dvl) is an important transducer in the Wnt-β-catenin signaling pathway. The binding of Wnt proteins to its membrane receptor Frizzled (FZD) leads to the activation of the phosphoprotein Dvl. Activated Dvl inhibits GSK-3β and AXIN, and prevents the constitutive destruction of cytosolic β-catenin. The accumulated β-catenin then translocates into nucleus to form a complex with T cell factor (TCF) and other transcriptional cofactors to induce the expression of downstream target genes. It has been indicated that the activity of Dvl can be suppressed by the interaction with nucleoredoxin (Nrx), a member of the Trxs family (101). Oxidative stress causes the dissociation of Nrx from Dvl and reactivates the Wnt downstream signaling pathway to promote cell proliferation. However, the role of ROS in the Wnt signaling pathway appears to be more complex. The dynamic change of ROS is important to the function of Wnt signaling pathway, because the action of β-catenin in response to intracellular redox state is dependent on the interaction with specific transcription factors (139). A moderate level of oxidative stress can stimulate the interaction of β-catenin with the FoxO transcriptional factor, which results in cell cycle arrest and a protective response to deal with the oxidative stress-induced damage (88). The consequences caused by the interaction of β-catenin with FoxO in response to oxidative stress are the suppression of β-catenin binding to the TCF transcriptional factors, and the subsequent alteration of cellular behaviors (140).

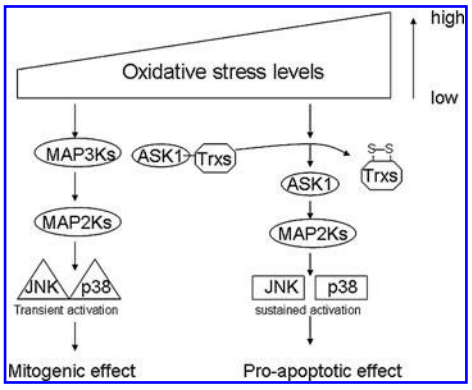

FIG. 4. Opposite effects of activated c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK) signaling in response to oxidative stress in determining cell fate and mediated by ASK1-Trxs complex. JNK, p38, as two MAPKs, can be transient activated by lower levels of oxidative stress, and induce mitogenic effect to promote cell proliferation or differentiation. However, the higher levels of oxidative stress can trigger a sustained activation of JNK and p38 signaling cascades, subsequently inducing a proapoptotic effect. The activity of ASK1, a MAP3K, plays a critical role during the sustained activation of JNK and p38. ASK1 is depressed by Trxs through interacting with the N-terminal regulatory domain of ASK1 to form an inactive complex. When the levels of oxidative stress are higher enough to oxidize Trxs, ASK1 can be activated and trigger the downstream signaling mediated by JNK and p38 in a sustained manner. So, ASK1-Trxs can be seen as the redox sensor of MAPK signaling cascades in determining cell fate, survival or death.

B. Key transcriptional factors regulated by ROS

Biological consequences caused by ROS are dependent on their amount, location, duration, and whether they overwhelm the antioxidant defensive systems. The direct oxidative modifications of the cellular component can activate multiple signaling cascades that sense and respond to cellular stress. In addition, some transcription factors can also be regulated according to intracellular redox states.

1. Nuclear factor (erythroid-derived 2)-related factor transcription factor. The initial response of cells under oxidative stress is the activation of defense systems that lead to a coordinate expression of a battery of defensive genes (77). In eukaryotic cells, over hundred gene products are involved in response to oxidative stress regulating a wide range of cellular activities. Nuclear factor (erythroid-derived 2)-related factors (Nrfs), known to be activated in response to various oxidative stimuli, belong to the family of basic leucine zipper proteins (bZIP). The Nrfs family has three members: Nrf1, Nrf2, and Nrf3. Nrf2 is presumably the most important transcription factor involved in a wide range of signal transduction pathways that deal with oxidative stimulation induced by the stress response (77). The first evidence demonstrating the roles of Nrf1 and Nrf2 in protection against oxidative stress came from studies on the role of Nrf2 in antioxidant-responsive element (ARE)-mediated regulation of NQO1 gene expression (383). Further studies showed that Nrf2 was also a prevailing factor in the regulation of ARE-mediated activation of other defensive genes, including *GST Ya*, γ-glutamyl cysteine synthetase (γ-*GCS*), and *HO-1* (5, 158, 402).

Nrf2 under normoxia conditions is retained in the cytoplasm by a specific cytosolic inhibitor, named inhibitor of Nrf2 (INrf2) or Kelch-like ECH-associated protein 1 (KEAP1) (155). Several reports have indicated that Nrf2 binding to INrf2 leads to degradation of Nrf2 (175, 348). When cells are exposed to oxidative stress induced by the oxidative species, Nrf2 dissociates from INrf2, stabilizes, and translocates into the nucleus, leading to activation of ARE gene expression. Several kinases, including protein kinase C (PKC), ERK, MAPK, p38 MAPK, and PRKR-like endoplasmic reticulum kinase (PERK), have been reported to activate Nrf2 by Nrf2 residue modification, thus releasing it from INrf2 (30, 157, 175, 265). Among these mechanisms, oxidative stress-mediated phosphorylation of Nrf2 at serine 40 by PKC is a very well-studied and accepted model for an activation mechanism of Nrf2 (30, 145). The cysteine residues Cys151, Cys273, and Cys288 of INrf2 are also crucial targets sensitive to redox modulation to exert an inhibitory effect on Nrf2 (418). In conclusion, an autoregulatory loop between stress sensors INrf2 and Nrf2 exists and controls their cellular abundance under normal conditions (204). Nrf2 protects cells against chemical and radiation stress and promotes cell survival. Loss or dysfunction of Nrf2 and INrf2 may cause cells to become more sensitive to oxidative damage, or induce the persistent activation of Nrf2, which imparts the survival advantage under oxidative conditions.

2. Nuclear factor-kappa B transcription factor. Nuclear factor-kappa B (NF-κB), an important inflammatory regulator, can be activated or inactivated by ROS. NF-κB as a transcriptional factor is responsible for activating its target genes involved in multiple cellular processes, including cellular survival, growth, differentiation, inflammation, and death. NF-κB can be activated by many stimuli and intracellular ROS levels are critical to the activity of NF-κB (169). It has been reported that moderate levels of ROS can activate phosphorylation and degradation of IKB, which plays a role in inactivation of NF-κB through sequestering it in the cytoplasm. Once activated, NF-κB can translocate into the nucleus to activate target gene transcription. Chromatin remodeling is a key event during transcription. The subunits that may comprise dimeric NF-κB are Rel B, C-Rel, p50, p52, and Rel A (p65) (242). Rel B and C-Rel are responsible for activation of transcription, and p50 and p52 possess DNA-binding properties. Rel A (p65) is known to participate in both DNA binding and transcription. It has been indicated that during NF-κB activation, ROS participate in phosphorylation of p65 ser^{276}, resulting in its interaction with the coactivator CBP/p300 and relieving the repressed promoters from histone deacetylase (HDAC) proteins (180). In addition, the reducing state is important for the DNA binding activity of NF-κB. When ROS are overproduced, they can inactivate the transcriptional activity of NF-κB at multiple steps, namely, through Grx-mediated S-glutathionylation of p65 subunit and oxidation of cysteine 62 at its p50 subunit, eventually inhibiting its DNA binding ability (242, 248, 365). Thus, ROS have a dual role in NF-κB transcriptional activity, and the on or off switching of target gene expression is determined by the specific conditions.

3. p53 transcription factor. p53 is an important transcription factor involved in DNA repair, cell survival, and cell death in response to a series of stress types, including oxidative stress. Many studies have indicated that p53 as a transcription factor can upregulate several antioxidant genes (351, 414), including glutaminase 2 (*GLS2*), *MnSOD,* and aldehyde dehydrogenase 4 (*ALDH4*), that will lower ROS levels, providing a survival function and protecting cells from oxidative injury. Indirectly, the multifunctional p53 also influences intracellular metabolic states as p53 promotes OXPHOS through regulating the expression of some components of electron transport chain (ETC) (233, 270). Simultaneously, intracellular redox states can also modulate the transcriptional activity of p53. It has been indicated that oxidation of p53 cysteine residues suppresses its DNA-binding activity (119). The dual role of p53 in cell survival and death indicates that p53 has a pro-oxidant effect on cell death. Some target genes of p53 such as p53 upregulated modulator of apoptosis (*PUMA*) and P53-induced gene 3 (*PIG3*) have a pro-oxidative role (222, 298). In colorectal tumor cells, PUMA overexpression induces apoptosis through Bax-dependent generation of superoxide and H_2O_2. PIG3 is a member of the dehydrogenase/reductase superfamily and is used as a proapoptotic marker. Its action in apoptosis can be determined by its enzymatic activity in ROS production (298). ROS accumulation to high levels can activate p53-mediated apoptosis through promoting the expression of a number of genes, including those encoding death receptors and proapoptotic members of the Bcl-2 family (221, 362). In addition to its role as a transcription factor in apoptosis, p53 has also been shown to exert its function in a transcription-independent manner. Indeed, endogenous cytoplasmic p53 can engage the apoptotic program directly in the absence of p53-induced transcription through activation of Bax to permeabilize mitochondria (58).

In addition, as the major source of intracellular ROS, the redox state of mitochondria is also important for p53 activation. Previous studies in normal lymphocytes and leukemia have indicated that mitochondrial components involved in OXPHOS contribute to the proapoptotic signaling of stress-induced activation of p53 (174). From studies in the skin carcinogenesis model, it was found that the activation and translocation of p53 to mitochondria preceded its action in the nucleus after the treatment of 12-O-tetradecanoylphorbol-13-acetate (TPA), a tumor promoting as well as an ROS-generating agent (424). In mitochondria, p53 interacts with MnSOD, the primary antioxidant enzyme of mitochondria, and subsequently decreases its enzyme activity, leading to a higher mitochondrial oxidation state, a decreased mitochondrial membrane potential ($\Delta\psi_m$), and subsequent apoptosis. Under nonstressed conditions, p53 is also present at basal levels in mitochondria and is important for the maintenance of mtDNA homeostasis. Evidence has emerged from a cell model of p53 knockout in mouse neonatal fibroblasts showing decreased mitochondrial transcription and downregulation of critical components necessary for maintaining the mtDNA copy number (202). Thus, it is speculated that the subcellular location of p53 in mitochondria may be a survival mechanism allowing cells to maintain normal mitochondrial function.

4. Hypoxia inducible factor-1 transcription factor. Hypoxia inducible factor 1 (HIF-1) is an important O_2-sensitive transcription factor, which adapts cells to decreased O_2 environment (329). HIF-1 is a heterodimer composed of HIF-1α and HIF-1β. The HIF-1β subunit is constitutively expressed in the cytoplasm independent of O_2 conditions, whereas the abundance of the HIF-1α subunit is modulated according to the O_2 level. The HIF-1α subunit harbors two transactivation domains called the N- and C-terminal transactivation domains. The N-terminal transactivation domain overlaps with a larger domain, denoted the O_2-dependent degradation (ODD) domain, which regulates HIF-1α protein levels in an O_2 concentration-dependent manner (117). Activation of HIF-1 is indicated by the translocation of HIF-1α to the nucleus and the dimerization of the two HIF-1 subunits, which in turn results in the binding of HIF-1 to its target genes that presumably contain regulatory motifs of hypoxia-responsive elements. The target genes are thought to be involved in vascular development, glycolytic metabolism, and also cell cycle events (203) (Fig. 5). Under normoxic conditions, HIF-1α appears to be modulated by post-translational hydroxylation of proline residues, resulting in rapid degradation of the protein through the pVHL-mediated ubiquitin-proteasome system (203, 316). Hydroxylation of proline residues is thought to be mediated by prolyl hydroxylase domain (PHD) enzymes that requires O_2 and 2-oxoglutarate as substrates, and Fe(II) and ascorbate as cofactors with a Fenton reaction, resulting in oxidation of two highly conserved proline residues P^{402} and P^{564}. As O_2 sensors, the K_m values of PHDs for O_2 are higher than normal tissue O_2 concentrations in normoxia, and the binding of O_2 to the iron center of PHDs active site supplies the essential condition for producing the highly reactive Fe(IV)=O intermediate that is responsible for HIF-1α hydroxylation (256). When cells are exposed to hypoxia, the degradation process is blocked and

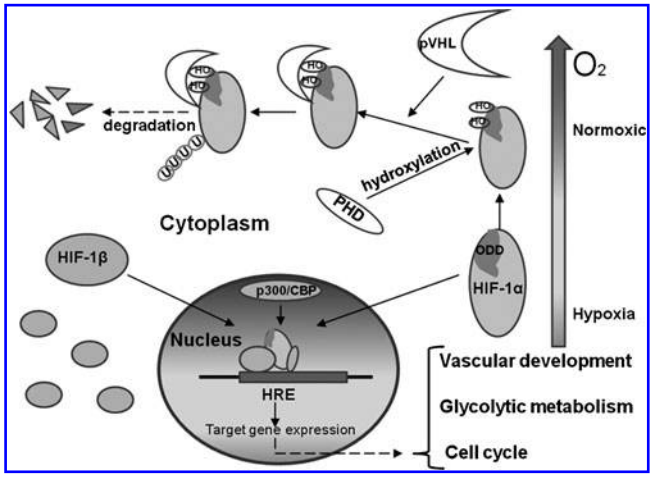

FIG. 5. Hypoxia-induced activation of hypoxia inducible factor-1α (HIF-1α). The O_2-dependent degradation (ODD) domain in HIF-1α contributes to its stabilization in diverse conditions. In normoxia conditions, PHDs mediate hydroxylation of proline residues in ODD subject to rapid degradation of HIF-1α by the process of pVHL-mediated ubiquitin-proteasome pathway. In response to hypoxia, the degradation process is blocked and HIF-1α accumulates in the cytoplasm and translocates into the nucleus to dimerise with the HIF-1β subunit, binding to p300/CBP and other coactivators to induce target gene expression to deal with hypoxia stress.

HIF-1α accumulates in the cytoplasm, and then translocates into the nucleus to dimerise with HIF-1β, and binds to p300/CBP and other coactivators to induce target gene expression. This is accomplished to deal with the stress of hypoxia (87), such as stimulating erythropoiesis and angiogenesis to increase O_2 availability, and switching the energy metabolic pathway to the O_2-independent glycolysis pathway, which is the primary mechanism of ATP production in hypoxia (304, 331). Besides the degradation of HIF-1α in normoxia, additional controls of HIF-1α include the asparagine hydroxylase factor inhibiting HIF (FIH-1), which hydroxylates Asp803 of the HIF-1 C-terminal transactivation domain and decreases the binding activity of HIF-1 to the transcriptional coactivator p300/CBP (195, 196). HIF-1α is overexpressed in many cancer patient samples and the significant association between HIF-1α overexpression and mortality in some cancer types is noteworthy (330). Further, it is thought that overexpression of HIF-1α is closely related to tumor metastasis and chemotherapy resistance (36, 217). Recent progress in the study of cancer stem cells (CSCs) has indicated that low-level O_2 conditions can increase the CSC fraction and induce cancer cells to acquire a stem cell-like state, a hypothesis that provides a new mechanistic explanation for the contribution of hypoxia to malignancy (131).

5. FoxO transcription factor. FoxO transcription factors, including FoxO1, FoxO3, FoxO4, and FoxO6, are characterized by the presence of a highly conserved DNA-binding region, termed a forkhead domain. FoxOs can regulate cell proliferation, cell death, and defense against oxidative stress, depending on the cellular environment (44). All FoxO transcription factors are inhibited by activation of the PI3K/Akt pathway (113). Insulin-mediated Akt activation can phosphorylate FoxOs, causing their exclusion from the nucleus, thus inhibiting their transcriptional effects. JNK also promotes nuclear translocation of FoxOs and induces the expression of FoxOs-dependent stress response genes (179). The phosphorylation of FoxO transcription factors by JNK results in the translocation of FoxOs from the cytoplasm to the nucleus. Several studies have provided a mechanism for FoxOs-mediated tissue or cell protection through activation of antioxidant, antiapoptotic, and autophagy genes during conditions of oxidative stress. In the nucleus, FoxOs can upregulate *MnSOD* and *CAT* gene expression, thereby triggering detoxification of ROS in response to stimuli that would normally cause ROS to accumulate (113). FoxOs also bind to coactivator or corepressor complexes and become acetylated or deacetylated, that is, acetylation by CBP/p300 and deacetylation by sirtuin 1(Sirt1), in response to oxidative stress. FoxOs can be activated by Sirt1 through deacetylation and induce antioxidant gene expression, exerting a stress-resistant effect (215).

C. Redox regulation of physiological processes

A number of physiological factors regulate or modulate the generation of ROS. In turn, ROS-inducible stress evokes many intracellular events, such as proliferation, differentiation, apoptosis, autophagy, aging, and mitochondrial biogenesis, thus representing crucial messengers in physiological processes.

1. Proliferation and differentiation. Multiple lines of evidence has underlined the role of ROS as signaling messengers in the regulation of cell proliferation in response to growth factor stimulation (361, 398). Notably, different levels of ROS can generate different cell fates. As aforementioned, low levels of ROS promote cell proliferation by transient stimulation of MAPKs, whereas a slight oxidative stress can cause cell growth arrest to obtain an adaptive response, including the expression of antioxidant as well as and DNA repair genes.

In addition, the redox states intracellular are important in cell differentiation because some related molecules can be regulated by ROS in direct or indirect manners. For example, the activity of the transcription factor Oct4 can be inhibited by oxidation, and Trx can physically associate with cysteines in the POU domain of Oct4 to protect it from oxidation (115). Recent studies in animal and plant models have highlighted a role of ROS in the control of cell differentiation. A lower level of ROS in hematopoietic stem cells (HSCs) as compared to the common myeloid progenitors has been found, and the significance of this ROS gradient to maintain stemness or differentiation has recently been demonstrated in a Drosophila model (276). The authors used *Drosophila* multipotent hematopoietic progenitors, which are similar to the mammalian myeloid progenitors, as a model to investigate changes in ROS levels during the differentiation process *in vivo*. They found that scavenging ROS from the progenitor cells retarded the process of differentiation into mature blood cells, and that upregulation of ROS above basal levels rescued the differentiation potential, implicating two regulatory mechanisms that may involve JNK and FoxO activation as well as Polycomb downregulation. Another good example in plants has demonstrated that the distribution patterns of ROS in different parts in the Arabidopsis root provided a transition between root cell proliferation and differentiation, and that this process was regulated by transcription factor UPB1, which directly regulates the expression of a set of genes encoding peroxidases (368). The evidence above suggests that a mild level of ROS may play an active part in cell differentiation processes.

2. Apoptosis. Apoptosis is the process of programmed cell death (PCD) that may occur in multicellular organisms involved in a variety of biological events. Two classical apoptosis-induced signaling pathways, known as intrinsic and extrinsic pathways, are particularly well studied. It is well established that ROS can induce cell death. Extracellular stimulation of death receptors can cause ROS generation and subsequent cell death. Death receptor signaling can be activated after extracellular stimulation with TNF-α, FasL, and TNF-related apoptosis-inducing ligands (TRAILs). The major known death receptors include Fas (CD95; APO-1), TNF receptor 1 (TNFR1), TRAIL receptor 1 (TRAIL-R1; DR4), and TRAIL receptor 2 (TRAIL-R2; DR5).

Here we have chosen to present the TNF-α-TNFR1 pathway as an example. TNF-α exerts its biological effects through binding to the TNFR1 superfamily of cell surface receptors, subsequently recruiting TNFR1-associated death domain protein (TRADD), TNF receptor-associated factor −2 (TRAF2), and the receptor interacting protein-1 (RIP1) to form the receptor complex, triggering its downstream signaling activation. ROS are generated as the second messengers involved in the signaling transduction of TNF-α-TNFR1 pathway (171, 250). A well-known pathway inducing apoptosis and activated by TNF-α is ASK1-mediated sustained activation of the JNK-MAPK cascades (364). As mentioned before,

ASK1 is a redox sensor in MAPK cascades and its activity is determined by the intracellular redox state. In addition, transient activation of JNK can also generate ROS, possibly by producing a positive feedback loop for TNF-α stimulation (382). Increased ROS production and sustained JNK activation are known to induce cell death. However, TNF-α does not usually cause cell death, possibly due to the activation of NF-κB transcription factor, another potent downstream signaling event (Fig. 6) (259, 281). ROS is known to participate in TNF-α-stimulated NF-κB activation. Several studies have indicated that activated NF-κB can suppress TNF-α-induced ROS accumulation and sustained JNK activation, suggesting an antiapoptotic effect (259, 281). It has been indicated that activation of NF-κB can induce the expression of several antiapoptotic proteins such as FLIPL, Bcl-xL, and X-linked inhibitor of apoptosis (XIAP) (241, 357). In addition, the antioxidant potential of NF-κB signaling may be due to the upregulation of ROS scavengers contained MnSOD and ferritin heavy chain (FHC) (75, 292).

ROS have also been shown to participate in the Fas-FasL-induced apoptosis signaling pathway. It has been suggested that NOX-dependent ROS generation can be activated to promote cell death through stimulation of Fas (352), whereas FLIPL, which functions as an antiapoptotic protein, can be downregulated through ROS-induced ubiquitination-proteasomal degradation (393). Increased intracellular glutathione levels can prevent Fas receptor-mediated apoptosis.

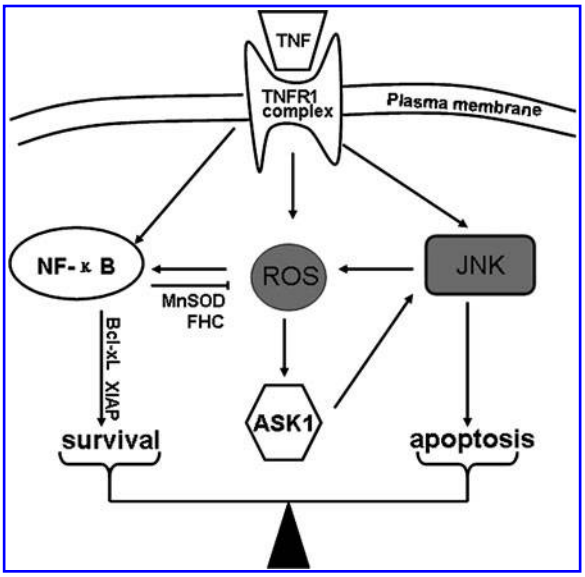

FIG. 6. Role of ROS in the balance between nuclear factor-kappa B (NF-κB) and JNK of tumor necrosis factor (TNF)/TNF receptor 1 (TNFR1) signaling. TNF binding to its receptor TNFR1 can trigger the death receptor signaling activation, and ROS can be generated as byproducts. NF-κB and JNK are two well-known targets of TNF/TNFR1 signaling. The sustained activation of JNK by ASK1 is responsible for the proapoptotic effect of TNF, and ROS perform a positive role to the activation of ASK1. The activation of JNK can also contribute to intracellular redox levels as a positive feedback. In another way, the activated NF-κB by TNF may prone to exert an antiapoptotic effect by the upregulation of some antiapoptotic genes (*Bcl-xL* and *XIAP*). Expression of antioxidant genes (*MnSOD* and *FHC*) by activated NF-κB can also modulate the redox levels and decrease the proapoptotic effect induced by TNF. So, the cell fate (survival or death) is determined by the balance between NF-κB and JNK signaling.

The intrinsic apoptotic pathway is triggered mainly by mitochondria through the formation of membrane pores or increased permeability of the outer mitochondrial membrane, causing the cytochrome c leakage. The released cytochrome c binds to Apaf-1 and procaspase-9 to form an apoptotic inducer that cleaves procaspase-9 into the active form to initiate the apoptosis pathway (112). The factors inducing mitochondrial dysfunction are multiplex, and the Bcl-2 family plays an important role in the regulation of outer mitochondrial membrane integrity and function. Mitochondria are not only responsible for energy generation; they also are the main source of intracellular ROS. The steady-state concentration of superoxide anion ($O_2^{\bullet -}$) has been estimated to be ~5- to 10-fold higher in the mitochondrial matrix as compared to the cytosolic or nuclear space (42). Thus, mitochondria are more vulnerable to damage caused by oxidative stress. It has been reported that ROS can trigger mitochondrial-induced apoptosis through affecting vital mitochondrial functions. This may occur by destroying the integrity of the respiratory chain to influence OXPHOS of ATP, decreasing the $\Delta\psi_m$, or impairing mitochondrial Ca^{2+} homeostasis (162). For example, release of cytochrome c from mitochondria is a crucial event in the apoptotic pathway, which can be regulated by ROS in oxidative stress-induced apoptosis. Cytochrome c is associated in the inner membrane of mitochondria with the anionic phospholipid cardiolipin (272, 274). One possible mechanism of oxidative stress-induced cytochrome c leakage is described below. Cardiolipin, which is responsible for the fluidity and stability of the mitochondrial membrane, can be peroxidated by ROS, resulting in the detachment of cytochrome C from the inner membrane (272, 274, 371). In parallel, the permeability of the outer membrane is regulated by members of the Bcl-2 family (275). Peroxidation of Cardiolipin and permeabilization of the outer mitochondrial membrane both contribute to mitochondrial cytochrome c release (272, 275).

In addition to their modulatory effect on the apoptotic signaling pathway, ROS can also directly alter the cell fate by modifying the activity of caspases that exert a controlling force in apoptosis. Caspases are a group of enzymes known as cysteine proteases, which exist in cells as inactive proforms or zymogens. Once apoptosis is triggered, these zymogens are cleaved to become active enzymes. During oxidative stress-induced apoptosis, members of the caspase family can be regulated by direct modifications of their Cys-containing active sites, through oxidation, glutathionylation, and S-nitrosylation.

3. Autophagy. Autophagy, as an adaptive response in unfavorable conditions, is a regulated lysosomal pathway responsible for the turnover of most of the macromolecules and organelles to maintain cellular homeostasis. Many factors can trigger autophagy, including nutrient-limiting conditions, environmental stress, and pathogen invasion (65).

Studies of signaling pathways involved in autophagy identified a series of autophagy-related genes (*Atg*). The *Atg* gene products function at several successive steps of this

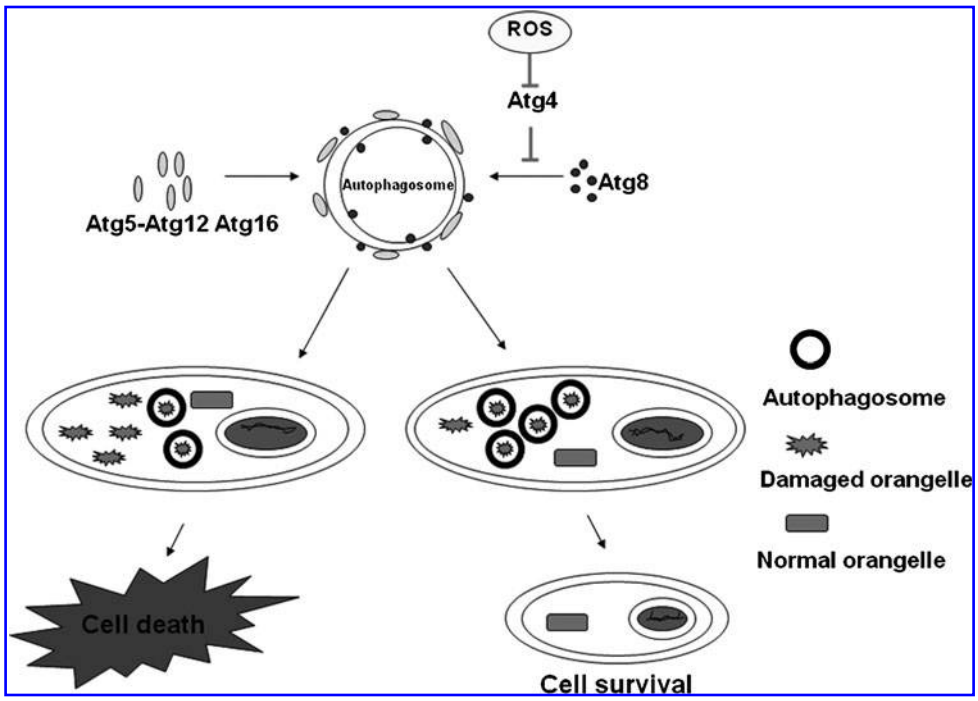

FIG. 7. Autophagy in response to ROS. Atg4 can be inactivated by ROS, ensuring the conjugation of Atg8s to the autophagosomal membrane. The formation of a tetramer of Atg12-Atg5-Atg16 complex by self-oligomerization is an essential step for the membrane elongation and expansion in forming autophagosome. Autophagy is thought to be the borderline between cell survival and death. Macroautophagy protects cells from destruction through removing the damaged organelles from cells, and cell death will not happen until the active autophagy is insufficient to remove the detriments.

pathway (including autophagy induction, cargo recognition and packaging, and vesicle formation and breakdown) and orchestrate much of these processes (130). Members of the Atg family include Atg4s that act both as conjugating and deconjugating enzymes with activities of cysteine proteases (116, 324). At least four *Atg4* mammalian homologs have been reported based on sequence homology to the yeast *Saccharomyces cerevisiae Atg4*. In the process of autophagy, after the initial cleavage of Atg8-like proteins, Atg4s will become inactive to ensure the conjugation of Atg8s to the autophagosomal membrane. Later on, as the autophagosome fuses with the lysosome, Atg4s are locally re-activated to delipidate and recycle Atg8s (268). Atg4s are cysteine-containing proteins, and thus are sensitive to the intracellular redox state. ROS target on the conserved residue Cys^{81} of Atg4s, which is in the vicinity of the catalytic Cys^{77} residue; oxidation of cysteines inhibits Atg4s protease activity and promotes lipidation of Atg8/LC3, an essential step for autophagy (17, 116, 130, 268). In addition, a recent report has indicated that another important protein named poly (ADP-ribose) polymerase-1 (PARP-1) can be activated through peroxidation, stimulating the LKB1-AMPK pathway and leading to autophagy induction (146).

Although it is well known that oxidative stress can induce cell death, there are, however, other intracellular pathways by which damaged macromolecules or organelles can be scavenged to allow cell survival. One of these is the protective macroautophagy. During the early stage of oxidative stress-induced damage, permeability of the mitochondrial membrane and its depolarization result in the leakage of intramitochondrial components such as cytochrome c, initiating apoptosis. Macroautophagy protects cells from destruction through removing the damaged mitochondria (116, 208), and cell death occurs if active autophagy is insufficient to remove the damaged material. Thus, this type of autophagy is thought to be on the borderline between cell survival and cell death as governed by the extent of oxidative stress (116, 337) (Fig. 7).

4. Aging. The physiological aging process is a normal event involving the progressive age-associated decline in tissue functioning. The mechanisms causing aging are complex and a large number of hypotheses have been proposed. One of the best accepted theories is the free radical theory of aging, which was first suggested by Denham Harman in 1956 (125), who postulated that free radicals generated endogenously by the utilization of O_2 to meet the energy needs can also cause tissue damage. An obvious example supporting this theory is the participation of ROS in the pathogenic processes of neurodegenerative diseases. Specifically, mitochondria are the main source of intracellular ROS, and mutations in mtDNA or dysfunction of ETC are correlated with aging (317). Mitochondria have been found to display age-associated damage in intact hepatocytes and mitochondrial glutathione oxidation in correlation with age-associated oxidative mtDNA damage (72, 319). Previous studies have also shown a negative correlation of antioxidants with maximum lifespan in different vertebrate species (223). It has been speculated that this correlation is an evolutionary result due to the low rate of O_2 radicals generated in longevous animals. In addition to the reduced generation of ROS, some stress-responsive signaling cascades may also participate during an extended lifespan. A recent finding indicates that *Klotho*, an aging-suppressor gene, can suppress the activity of the ROS-responsive ASK1-signalosome and its subsequent p38 MAPK signaling in a *Klotho* overexpressing mouse model (141).

Administration of antioxidants to prevent the oxidative damage associated with aging and age-related diseases has been widely studied *in vitro* and *in vivo*. Recent studies in rats indicate that supplementation of food with folic acid can decrease the aging rate of brain through reduction of oxidative stress and maintenance of the integrity of neurons (340).

However, these results are still controversial, as other experimental data do not support this hypothesis. For example, in the genetically modified mice with partial deficiency of SOD2 (SOD2 + / −), no influence on longevity was shown, whereas the animals did exhibit increased oxidative damage and a proneness to cancer (380). Multiple clinical trials also indicate that administration of antioxidants to reduce the generated ROS has no effect on the aging rate but has an effect on decreasing oxidative stress-induced damage in pathological situations (27).

5. Mitochondrial biogenesis. Mitochondrial biogenesis is the process by which new mitochondria are formed in the cell. Mitochondria that contain double membranes and several hundred proteins as well as 2–10 copies of mtDNA in the matrix are responsible for biological oxidation in cells. Their principal function is to synthesize ATP through the respiratory chain, or electron transport and OXPHOS. Mitochondria are also the main intracellular source and immediate target of ROS, and accumulation of ROS can affect mitochondrial biogenesis to a large extent. For example, in some aging tissues, the mtDNA copy number was found increased (19, 289). One explanation for such an observation is the fact that an increase in mtDNA copy number is needed to compensate for the oxidative stress-induced damage to the respiratory chain. The peroxisome proliferator-activated receptor gamma (PGC) family of transcriptional coactivators, including PGC-1α, PGC-1β, and the PGC-related coactivator, are thought to be master regulators of mitochondrial biogenesis and essential for the expression of genes involved in aerobic metabolism (300). Activation of PGC-1 can be regulated by the oxidative stress-responsive signaling pathways such as p38 MAPK (299). In addition, PGC-1 can also contribute to the antioxidant system in mitochondria and protect mitochondria from oxidative damage. For example, PGC-1α regulates the expression of myocardial mitochondrial antioxidants SOD2 and Trx2, and protects heart tissue against the myocardial oxidative stress induced by chronic systolic pressure overload after transverse aortic constriction (224). In summary, ROS can increase mitochondrial abundance and mtDNA copy number in human cells. Moreover, the increase of mitochondria contribute to more ROS generated in cells, amplifying the oxidative damage to mitochondria and other intracellular constituents such as DNA, RNA, proteins, and lipids, to finally initiate the process of aging or apoptosis.

D. Regulation of cell survival and death by NOXs under normal physiological conditions

Numerous studies have demonstrated that under normal physiological conditions, NOXs are major redox regulators in a variety of cell activities or functions, including cell survival and death. Various stimuli such as transforming growth factor β (TGF-β), angiotensin II (AGT), and PDGF can regulate the expression of the NOXs, and in some conditions NOX-generated ROS have also been shown to induce genes such as *TNF*-α, *TGF*-β*1,* and *AGT* through regulating the redox-sensitive transcription factors NF-κB and AP1 (38). NOXs family members have different tissue distributions and subcellular locations, indicating that they may participate in different cell functions. In most circumstances, activation of NOXs is associated with cell death (23, 220). For example, NOX2-derived ROS play essential roles in innate host defense through ROS-dependent killing of the invaders after phagocytosis. However, a prosurvival function of NOXs has also been demonstrated in recent studies. For example, the activation of NOX-generated ROS by Ras promotes both the survival and the growth factor-independent proliferation of CD34(+) cells (136). NOX4 is the major isoform of NOXs in response to TNF-α-induced apoptosis in cerebral microvascular endothelial cells (CMVEC), and recent studies have shown that NOX4-generated ROS can also promote CMVEC survival through increased production of a gaseous antioxidant mediator, carbon monoxide, resulting in the inhibition of TNF-α-induced ERK1/2 and p38MAPK activities in an Akt-dependent manner (21). Angiogenesis is another important event regulated by NOX-generated ROS. Both the small GTPase Rac1 and gp91 (phox) are critical components of the endothelial NOXs complex as the major source of ROS in endothelial cells (EC). NOX-derived ROS can activate the vascular endothelial growth factor (VEGF) signaling pathway and induce angiogenesis by stimulating EC proliferation and migration. For example, after chronic load-induced stress in cardiomyocytes, NOX4 is upregulated, and promotes stress-induced activation of HIF-1 and the subsequent release of VEGF, resulting in increased paracrine angiogenic activity (421).

IV. Abnormal Redox Balance in Diseased Cells

ROS are biphasic, by acting as messengers in the regulation of signal transduction under normal conditions or as mediators in the pathogenesis of many diseases. As mentioned above, when ROS accumulate and cannot be detoxified by antioxidant agents, they can do damage to cellular components, giving rise to senescent, degenerative or fatal cell lesions. An excessive increase in ROS production has been implicated in the pathogenesis of atherosclerosis, ischemia/reperfusion injury, DM, neurodegenerative diseases, and cancer.

A. Atherosclerosis

Atherosclerosis is characterized by the accumulation in the arterial intima of mainly low-density lipoprotein (LDL)-derived lipids along with apolipoprotein B-100 (apoB100). The majority of cardiovascular diseases are the result of complications caused by atherosclerosis. Atherosclerosis is a multifactorial pathology whose molecular etiology involves the interaction of many gene products and environmental factors. Much evidence has indicated that the injury and dysfunction of the endothelium contribute to the initiation and development of atherosclerotic lesions (386, 387). ROS, which appear to participate in the pathogenesis of a variety of cardiovascular diseases, have been reported to be involved in the damage to endothelium. Besides, increased levels of modified LDL as well as the initiation of the inflammation response during the atherosclerotic process are also related to the overproduction of ROS (1, 386, 387, 389).

1. Source of ROS in the progression of atherosclerosis. Among the enzymatic pathways involved, membrane-bound NOXs are the most important enzymes responsible for ROS generation in human vessels (127, 389). In particular, NOXs are responsible for the formation of superoxide anion. Much clinical evidence has shown that an enhanced

expression and activity of NOXs is associated with atherosclerosis, suggesting that these enzymes may participate in the initiation and progression of atherosclerotic disease (389). Pathological stimuli such as inflammatory cytokines, Ang II, endothelin-1, thrombin, and catecholamines acutely activate the NOXs in vascular smooth muscle cells (VSMCs) and endothelial cells (37, 315, 375). In addition, accumulation of mtDNA damage and progressive respiratory chain dysfunction causing increased production of ROS in mitochondria are also associated with atherosclerosis in human and animal models of oxidative stress (386). Another important source of ROS is from XO (194, 386). Under conditions of atherosclerosis, XO can produce ROS and affect endothelial cell function through stimulation of XO expression by inflammatory cytokines or other stimuli. Evidence has been provided that feeding apolipo apolipoprotein protein E deficient ($ApoE^{-/-}$) mice with a Western-type diet upregulates the XO content and leads to the development of atherosclerosis, and that XO inhibitors can prevent this process (326).

2. Endothelial dysfunction and inactivation of endothelial nitric oxide synthase. Endothelial dysfunction is an early key event in atherosclerosis (296, 387) and ROS are likely to be involved in the progression of endothelial cell dysfunction. Under normal conditions, vascular endothelial cells, through regulating a variety of functions, including vascular smooth muscle tone, host-defense reactions, angiogenesis, and tissue fluid hemostasis, play important roles in the inflammatory and proliferative response to injury. Dysfunction of endothelial cells can induce a series of disorders characterized by an impairment of secretory substances with a minor liberation of vasodilator substances, such as nitric oxide (NO), anticoagulants and platelet inhibitors, and an increase of the proinflammatory molecules, thus inducing a procoagulant, antifibrinolytic, vasoconstrictor, and proinflammatory condition (293, 386, 387).

During the process of endothelial dysfunction, a concomitant decrease of NO has been reported. It is known that NO is a potent vasodilator and antiaggregating molecule produced by endothelia that regulates vascular permeability and the vasomotor tone in endothelial cells. Endothelial nitric oxide synthase (eNOS), an enzyme that is constitutively expressed in endothelia, is involved in NO formation. It catalyzes electron transport from NADPH to a prosthetic heme group. This process appears to require tetrahydrobiopterin (BH4) to complete O_2 activation and transfer to a guanidine nitrogen of L-arginine to form NO (97). eNOS is one of the potential sources of ROS. In turn, over-produced ROS can result in oxidation of BH4 to dihydrobiopterin (BH2) and the Zn^{2+} thiolate cluster in eNOS, leading to enzyme uncoupling (Fig. 8) (97). ROS can induce inactivation of NO through direct reaction with NO and the formation of the highly reactive species peroxynitrite ($ONOO^-$) (278, 305).

3. LDL oxidation and inflammation triggers. Two critical events in the pathogenesis of atherosclerosis are the accumulation and oxidation of LDL in the arterial intima and the triggering of inflammation responses. LDL accumulates in the arterial intima when its rate of influx exceeds the rate of efflux. Once in the arterial intima, LDL can be oxidized to Ox-LDL through oxidation of polyunsaturated fatty acid (PUFA), particularly phosphatidylcholine (PC) of LDL to form Ox-PC (235). The Ox-LDL is harmful to normal endothelial cell function. These oxidized lipids contribute to lesion progression through their profibrinogenic, proapoptotic, procoagu-

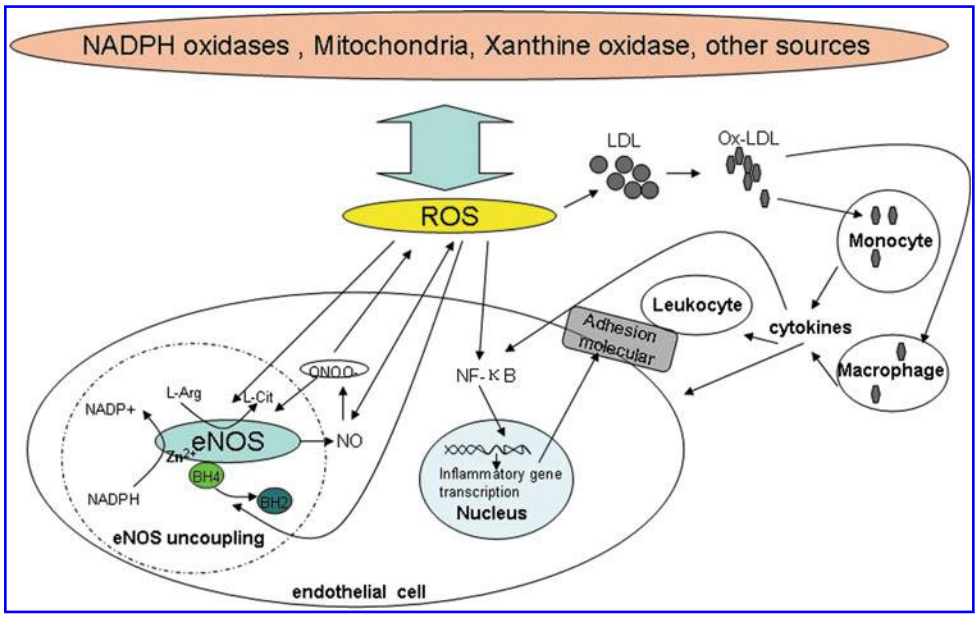

FIG. 8. Pathological roles of ROS in endothelial endothelial nitric oxide synthase (eNOS) inactivation and inflammatory triggers. ROS mainly derived from mitochondria, NOXs, and XO contribute to endothelial dysfunction. One of the most important events during endothelial dysfunction is eNOS uncoupling caused by over-loaded ROS. eNOS catalyzes electron transport from NADPH to a prosthetic heme group and this process appears to require tetrahydrobiopterin (BH4) to complete the activation of O_2 and transfer to a guanidine nitrogen of L-arginine to form NO. ROS can disrupt the reaction through oxidizing BH4 (to BH2) and the Zn^{2+} thiolate cluster, resulting in NO decrease. ROS can also directly react with NO to form a high radical species $ONOO^-$, which also induce eNOS uncoupling. The aggregated low-density lipoprotein (LDL) in arterial intima can be oxidized by ROS and produce a series of detrimental ox-LDL. Ox-LDL can be phagocytized by macrophages and monocytes, leading to the release of various inflammatory cytokines. Oxidative stress and inflammatory cytokines could activate redox-sensitive transcription factors such as NF-κB and induce endothelial expression of adhesion molecules, which is essential for the adhesion of leukocytes. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

lant, and proinflammatory effects. For example, HNE, an aldehyde end product of lipid peroxidation, has been detected at high levels in atherosclerotic lesions, which may be responsible for its proatherogenic effect (211, 328). Previous work has provided an indication that the increased level of HNE enhances matrix metalloproteinases-2 production in VSMCs through mitochondrial ROS-mediated activation of Akt-linked NF-κB pathways (205). In atherosclerotic lesions, the increase of matrix metalloproteinases (MMPs) is responsible for the rupture of atherosclerotic plaques and promotes the pathological changes (176). Advanced glycation end-product receptor (RAGE), which is the receptor of advanced glycation end-products (AGE), can also be activated by Ox-LDL and subsequently induce ROS generation by a variety of mechanisms (96, 388). Activation of RAGE may alter the response of vessels to mediators of tone by contributing to the phenotypic changes and remodeling associated with vascular disease and possibly due to altered bioavailability of the labile vasodilator NO (96). Additionally, the subsequent ingestion of excess Ox-LDL particles by macrophages and monocytes leads to the release of various inflammatory cytokines and growth factors (325) (Fig. 8). NF-κB is also an important transcription factor involved in the inflammation process. It has been reported that Ox-LDL can activate NF-κB and upregulate proinflammatory gene expression (325). In addition, the inflammatory response can also promote the secretion of myeloperoxidase (MPO), a member of heme-containing peroxide enzymes that is released from phagocytes and catalyzes a reaction between H_2O_2 and chloride to form a two-electron oxidant hypochlorous acid. MPO-derived chlorinated biomolecules can oxidize nitrite to form reactive nitrogen species (RNS), which exert a role in LDL oxidation and protein modifications, enhancing the role of oxidative stress in endothelial dysfunction (199).

Adhesion of leukocytes to the vascular endothelium is the pivotal early event in atherogenesis. Inflammatory cytokines stimulate the endothelium and can activate redox-sensitive transcription factors such as NF-κB, which induce the expression of adhesion molecules (186, 232) (Fig. 8). ROS participate in the expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), which are responsible for inflammatory cell adhesion on the endothelial surface, their release of cytokines and chemokines that mediate the inflammation response, VSMC proliferation, and thrombogenesis in vessel walls and promote atherosclerotic lesions. This process can be partially inhibited by different antioxidants, which suggests a potential role of endogenous ROS in the inflammation response during atherogenesis.

4. ROS-induced VSMC proliferation. VSMC proliferation is an important event during the development of atherosclerotic lesions. For many years, the role of ROS in the remodeling of the vasculature has been investigated (320), and today it is relatively clear that ROS play a crucial role in VSMC proliferation by inducing both direct and indirect auto/paracrine growth mechanisms (360). Recently, it has been found that an important factor, cyclophilin A (CyPA), can regulate the proliferation of VSMCs through auto/paracrine mechanisms (320). Cyclophilins are a family of highly conserved and ubiquitous proteins termed immunophilins. ROS can induce the expression and secretion of CyPA, which then stimulates ERK1/2, Akt, and JAK in VSMCs contributing to DNA synthesis, cell proliferation, and migration (165).

B. Ischemia and reperfusion injury

Ischemia can be described as an inadequate flow of blood in the body caused by vasoconstriction, thrombosis, or embolism. Since O_2 is mainly bound to hemoglobin present in red blood cells, insufficient blood supply causes tissues to become hypoxic, or, if no O_2 is supplied at all, anoxic. In very aerobic tissues such as heart and brain, necrosis due to ischemia usually occurs in about 3–4 mins at body temperature, before becoming irreversible. Reperfusion injury refers to damage to tissue caused when blood supply returns to the tissue after a period of ischemia. Many studies have indicated that the process of restoration of the circulation, rather than having a curative effect, can do great damage to the ischemic tissues or organs. Although ischemia and reperfusion injury are related to a variety of diseases such as cardiovascular and autoimmune rheumatoid diseases, the mechanisms are still unclear. Today, it is widely accepted that the injury caused by ischemia-reperfusion is partly due to an inflammatory response and an overproduction of ROS (214, 395). A role for ROS during ischemia-reperfusion-induced injury is indicated by the observation that a variety of antioxidant strategies have been shown to reduce cardiomyocyte cell death (287, 395).

1. Role of ROS in ischemia-induced metabolic change. During the ischemia process, low levels of ROS have been observed. However, the pathological significance is unclear. The injury induced by ischemia differs according to duration. It has been reported that cells may have the ability to tolerate a brief exposure to ischemia as the inherent mechanisms to preserve energy levels also prevent injury (55, 247). Low level production of ROS may be important in protective signaling activation strategies. These include a switch of the metabolism to anaerobic glycolysis and fatty acid utilization, increased glucose uptake, and decreased ATP consumption. This adaptive response to brief ischemia has been well studied in view of the development of protective strategies in tissue transplantation, a procedure called preconditioning (255). However, when the ischemia persists, the damage to cells is irreversible and eventually induces cell death (78, 213). During this process, ROS have a negative impact and large amounts of ROS are produced.

2. Cell damage induced by reperfusion-induced ROS. The initial injury caused by reperfusion of blood is always accompanied with a burst of ROS, in addition to the increased cytosolic free Ca^{2+}. It has been suggested that ROS generated during early reperfusion is the primary activator of the inner mitochondrial large-conductance channel known as the mitochondrial permeability transition pore and of cardiomyocyte death (209). During reperfusion, the rise in free cytosolic Ca^{2+}, NADPH oxidation, decrease in adenine nucleotide levels, and increase in pH all contribute to the activation of mitochondrial permeability transition pore, which induces ATP decline and mitochondrial dysfunction (120). These conditions favor the activation of mitochondrial permeability transition pore, which leads to depolarization of $\Delta\psi_m$, uncoupling of OXPHOS, and decrease of mitochondrial

energy production. In an attempt to maintain $\Delta\psi_m$, F1F0ATPase can reverse its activity by hydrolyzing ATP and contributing to a further decline in ATP levels with loss of metabolic homeostasis, activation of various enzymes, and cell necrosis. If the mitochondrial permeability transition pore is activated together with proapoptotic Bax, cytochrome c can be released from the mitochondrial intermembrane space and lead to the induction of apoptosis. Thus, the mitochondrial permeability transition pore appears to be a sensor of ROS levels and functions as an important regulator of cell death in ischemia-reperfusion-induced injury. New protective strategies to inhibit the activation of mitochondrial permeability transition pores will result most likely in a significant reduction in infarct size during ischemia-reperfusion.

3. Source of ROS in pathogenesis of ischemic-reperfusion. In addition to leakage of electrons from the mitochondrial ETC, another potential source of reactive O_2-derived free radicals in reperfused tissue is XDH/XO (22, 247). In normal tissue, XDH is the major intracellular enzyme using NAD as electron acceptor for hypoxanthine and xanthine oxidation. The conversion of XDH to XO occurs during tissue ischemia (51). When molecular O_2 is readmitted during reperfusion, XO can use molecular O_2 as electron acceptor to generate a large amount of superoxide anion ($O_2^{\bullet -}$). Although it is acknowledged that ROS are important contributors to tissue damage during the ischemia-reperfusion process, the translation of this concept to clinical therapy is difficult. Many clinical studies have failed in utilizing antioxidant substrates or enzymes to reduce infarct size, a result that may be due to the inability of these antioxidants to act at the right time and location, given the fact that injury induced by ischemia-reperfusion typically happens within minutes.

C. Neurodegenerative diseases

Neurodegenerative diseases are a heterogeneous group of disorders represented by Parkinson's disease (PD), Huntington's disease (HD), and Alzheimer's disease (AD), and characterized by a gradual and selective loss of anatomically or physiologically related neuronal cells. Although the phenotypes of these diseases are heterogeneous, ample evidence indicates that mitochondrial dysfunction and overloaded ROS occur early in their pathogenesis, suggesting a causal role in the disease development (283). For example, in AD, oxidative stress occurs early in AD brain, before the onset of significant plaque pathology (267), and induces early mitochondrial metabolic disorders and apoptosis. The direct evidence for the participation of overloaded ROS is that the antioxidant enzyme SOD1 is mutated in cases of familial amyotrophic lateral sclerosis (FALS) (313). Dysfunction of SOD1 induces not only disrupted antioxidant function, but also mutant protein aggregation in mitochondria, which then cause electron transfer chain impairment, ROS production, and mitochondrial dysfunction (427). In HD transgenic mice, ROS have been suggested to play an active role in the onset and progression of the neurological phenotype. In addition, in PD and AD, an overload in ROS has been widely reported, and several critical proteins involved in the pathogenesis of neurodegenerative diseases are related to mitochondrial functions and oxidative stress (10, 69, 219, 339).

1. ROS in the pathogenesis of AD. AD is the most common form of dementia among elderly people. Senile plaques and neurofibrillary tangles are the histological hallmarks of AD and two of the most probable causes of AD pathogenesis. Amyloid precursor protein (APP) cleaved by β- and γ-secretases produces amyloid-β peptide (Aβ), which is the primary component of senile plaques. APP is important in the pathogenesis of AD and transgenic mice that express a mutant form of the human APP gene develop fibrillar amyloid plaques and Alzheimer's-like brain pathology with spatial learning deficits. APP has been found to have a dual ER/mitochondrial-targeting sequence, and in transfected cells or in transgenic mice overexpressing APP, this protein clogs the mitochondrial protein importation machinery, causing mitochondrial dysfunction and impaired energy metabolism (10). The accumulated APP in mitochondrial import channels inhibits the import of nucleus-encoded cytochrome c oxidase subunits and subsequently impairs ETC, causing increased levels of ROS (76). Mitochondrial dysfunction is a major intracellular lesion in AD. Alterations in mitochondrial energy metabolism and in mitochondrial dynamics (mitochondrial fission/fusion, autophagy, *etc.*) have been found in the early stage of AD (84). Accumulation of Aβ in synaptic mitochondria was detected in the early stage before the onset of nonsynaptic mitochondria. The impairment of synaptic mitochondria by accumulation of Aβ contributes to mitochondrial dysfunction and synaptic degeneration, which are early pathological features of AD (82). Although detailed mechanisms for Aβ in mitochondrial dysfunction are not fully understood, it is admitted that the overloaded Aβ in mitochondria results in more free radicals and oxidative damage to mitochondria (227, 306). Also, some other reports have suggested that Aβ could interact with mitochondria to disrupt cytochrome oxidase activity and increase free radical generation (63, 227).

In addition, a mild but persistent inflammation *via* the activation of microglia and astrocytes occurs in the early pathogenetic stage of AD. During this inflammation process, ROS generated through the overexpressed NOXs in microglia promote the production of proinflammatory and neurotoxic cytokines that amplify the inflammation, in response to both Aβ accumulation and neuronal damage (29). Aβ accumulation also contributes to the generation of NO through activation of the inducible form of nitric oxide synthetase (iNOS), and overgenerated ROS react with NO to form the highly reactive species $ONOO^-$ and subsequently cause oxidative damage to the neuronal cells (18). Simultaneously, the sequestration of NO by ROS impairs the resting cerebrovascular tone and NO-dependent dilatations, resulting in cerebrovascular dysfunction (122). In AD, the vascular pathology is characterized by accumulation of Aβ in the vessel wall, atherosclerosis, vascular fibrosis, and structural and inflammatory changes of blood vessels, causing chronic cerebral hypoperfusion. It has been suggested that the perfusion defect may occur ahead of the neurodegenerative changes and contribute to the pathogenesis of AD (73).

As mentioned above, Aβ is toxic to mitochondria and induces free radical species which subsequently cause the release of proapoptotic factors. The Aβ-binding alcohol dehydrogenase (ABAD) localizes in mitochondria and can interact with Aβ. Neuronal cells cultured from transgenic mice overexpressing a mutant form of APP and ABAD

display a spontaneous generation of superoxide anion and H_2O_2, eventually inducing mitochondrial dysfunction and cell apoptosis (227). Some studies suggest that blocking the interaction of Aβ and ABAD with a "decoy peptide" could suppress Aβ-induced apoptosis and free-radical generation in neuronal cells (225, 412).

2. ROS in the pathogenesis of PD. PD is characterized clinically by progressive rigidity, bradykinesia, and tremor, and pathologically by loss of pigmented neurons in the substantia nigra and the presence of Lewy body-distinctive cytoplasmic inclusions that immunostain for α-synuclein and ubiquitin (219). The discovery of overloaded ROS in PD came from the observation that human PD brains show signs of mitochondrial dysfunction and oxidative damage in degenerating areas, including the substantia nigra (6, 327). Models that use ETC complex I inhibitors, 1-methyl 4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) or rotenone, can induce a parkinsonian phenotype (25, 100). Identification of the PD-related genes participating in mitochondrial function also confirms the role of ROS and mitochondria in the pathogenesis of PD. It has been suggested that these mitochondria related proteins may form a functional complex which has the role in stimulating a proteasomal degradation process to prevent accumulation of neurotoxins. For example, PTEN-induced kinase 1 (PINK1) is a kinase localized in mitochondria. A mutated PINK1 has been found in the form of autosomal recessive juvenile PD (376). Although the function of PINK1 in PD pathogenesis is still unclear, there has been some evidence showing that its role is associated with cell protection against oxidative stress. Datum from the cell model with PINK1 loss-of-function have revealed mitochondria-derived ROS upregulation, cristae/respiratory dysfunction, and calcium homeostasis destabilization, which triggered compensatory fission, autophagy, and biosynthetic repair pathways that dramatically altered mitochondrial structure (311). DJ-1 is another mitochondria-related protein involved in cell protection against oxidative stress and its mutation is also related to a portion of the PD population (32). Parkin encodes for an ubiquitin E3 ligase and localizes in mitochondria to protect them from damage especially caused by oxidative stress. Its mutation can induce abnormalities in the proteasome system, which may increase ROS levels and induce dopaminergic neurons apoptosis (401). Besides dysfunction of the proteasome system caused by ROS, the aggregation of toxic proteins such as mutant α-synuclein also induces mitochondrial damage and ROS overproduction, eventually inducing cell death (231). On the other hand, mitochondrial dysfunction induces ROS upregulation and subsequent protein oxidation, and in parallel the impairment of ETC causes ATP deficiency and subsequent microtubule depolymerization, thus contributing to the α-synuclein oligomerization in PD cybrids (89).

Similar to AD, stimulation of microglia and astrocyte activity may contribute to the inflammatory response to the exposure of neurotoxins and subsequent brain damage as well. Microglia behave as macrophages in the brain, and they play an active role in free radical generation by activating NOXs and iNOS signaling (244). During the pathogenic processes of PD, the metabolic changes in neuronal cells contribute to oxidative stress as well (57). In the early stage of PD, dopamine, an important neurotransmitter, can be increased to compensate the dying dopaminergic neurons (323). However, it has been found that the antioxidant activity in dopaminergic neurons of diseased brain is decreased, partially by the decreased GSH level and increased iron level, and dopamine is prone to undergo auto-oxidation to form dopamine quinones and superoxide anion radicals (177, 284).

D. Diabetes mellitus

DM is a chronic metabolic disorder characterized by a high blood glucose level, either because the body does not produce enough insulin (type 1 DM), or because body cells do not properly respond to insulin that has been produced (type 2 DM). Type 2 DM is the most common type representing $\sim$90% of DM cases in North America and Europe.

Since DM is incurable, patients need administration of drugs or injection of insulin to maintain the blood glucose at a suitable level to avoid complications. The pathogenesis of DM is complex and is influenced by both genetic factors and environmental elements. Among these, increased oxidative stress and free radical-induced damage have been proposed to be implicated, and several conditions leading to ROS generation in diabetes have been established (173, 216). Both hyperglycemia and lipotoxicity can induce the dysfunction of mitochondria, resulting in increase of ROS produced by the damaged ETC. The membrane-bound NOXs are also an important source of ROS and can be activated in DM by various stimuli such as AGEs, insulin, and Ang II. Under conditions of hyperglycemia, the formation of AGEs is markedly accelerated. During this process, the production of ROS is also highly increased.

To date, more and more evidence supports the contributory role of ROS in the pathogenesis of DM. In type 1 DM, ROS participate in β-cell dysfunction initiated by autoimmune reactions and inflammatory cytokines (301). In type 2 DM, excessive ROS impair insulin synthesis and activate β-cell apoptotic pathways (93, 126). Markers of oxidative damage, 8-hydroxy-2′-deoxyguanosine (8-OHdG) and HNE, are found to be increased in islets under diabetic conditions (109, 151). It has also been reported that exposure to ROS of β-cell-derived cell lines or isolated rat islets can lead to insulin mRNA expression decrease, which might be caused by the suppression of transcription factors that are responsible for the induction of the insulin genes by ROS (126, 234). Antioxidant treatment with N-acetyl-L-cysteine plus vitamin C and E retained glucose-stimulated insulin secretion and moderately ameliorated glucose tolerance in obese diabetic C57BL/KsJ-db/db mice (172). Evidence for systemic oxidative stress *in vivo* includes detection of increased circulating and urinary levels of the lipid peroxidation product F2-isoprostane in both type I and type II diabetic patients (70, 71).

Accordingly, dysfunction of pancreatic β-cells and insulin resistance are the two most important aspects during the development of type 2 DM, and ROS are reported to participate in these processes through their excessive production.

1. Role of ROS in the dysfunction of pancreatic β-cells. Pancreatic β-cells are sensitive to changes in the blood glucose level and responsible for regulation of insulin secretion, thereby maintaining glucose homoeostasis. In DM, the toxicity of chronic hyperglycemia causes β-cell dysfunction through induction of oxidative stress and lipotoxicity. The

electron transduction chain in mitochondria is the major source of ROS in β-cells (261). In addition, there is an independent mechanism responsible for generation of ROS in β-cells, which involves activation of the membrane-associated enzymes NOXs (261).

GLUT2 is a high-Km glucose transporter in β-cells, and under conditions of chronic hyperglycemia, the large uptake of glucose induces ROS overproduction, and simultaneously, the relatively low content of antioxidant enzymes in β-cells makes the cells unable to scavenge harmful substances that consequently induce oxidative stress.

Pancreatic and duodenal homeobox 1 (PDX1) and v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA) are two critical transcription factors that regulate insulin expression. ROS overproduction can suppress the binding of PDX1 and MafA to the insulin genes, and thus inhibit its expression (126, 173, 234). Further, activation of the JNK pathway caused by oxidative stress is involved in reduction of insulin genes expression. In DM, the activated JNK pathway inactivates PDX1 through sequestering it in the cytoplasm, away from its targets in the nucleus (178). One study has further indicated that the role of the JNK pathway in suppressing PDX1 might be mediated by increased amounts of nuclear FoxO1, the active form of another critical transcription factor capable of sensing the degree of intracellular ROS (179). Consistent with these findings, it has been shown that jun D proto-oncogene (JunD), a transcription factor of the AP1 family, plays an important role in inhibiting the insulin signaling pathway and activating Foxo-1, through ROS-dependant mechanisms (200).

The deleterious effects of lipotoxicity are also important in the dysfunction of β-cells. A link between free fatty acids (FFA) and ROS production in β-cells has been described in the late 1990s (46). Chronic treatment of rat islets with palmitate increased ROS production, although the mechanism of this process is still unclear. Exposure of β-cells to FFA can also activate iNOS, generating large amounts of intracellular NO, which can be related to β-cell apoptosis in DM (336).

2. Role of ROS in insulin resistance. Under normal physiological conditions, insulin binds to its receptors on the cell surface and induces phosphorylation of the insulin receptor and its substrates, leading to activation of various insulin signaling pathways. During this process, ROS play an active role in the insulin signaling pathways. Studies in adipose cells and in muscle cells have shown the ability of ROS to enhance insulin signaling (129, 239). For example, insulin-mediated stimulation of NOX4 activity induces ROS generation, which can suppress the negative regulator of the insulin signaling cascade, PTP, nonreceptor type 1 (PTP1B), the major intracellular tyrosine phosphatase (20).

Under diabetic conditions, various insulin-targeted tissues such as the liver, muscle, and adipose tissue become resistant to insulin. The pathophysiology of insulin resistance involves a complex network of insulin signaling pathways. More recently, ER stress was suggested to participate in insulin resistance, and critical markers of ER-stress were detected in some insulin resistance tissues (260, 416).

Further, in type 2 DM patients, both acute and chronic administration of the antioxidant α-lipoic acid was able to improve insulin resistance, suggesting an important role of ROS in insulin resistance. The mechanism by which ROS participate in insulin resistance is still unclear. However, ROS-induced activation of the JNK pathway may play an important role in insulin resistance in liver, muscle, and adipose tissue cells. The activated JNK pathway can induce the serine phosphorylation of insulin receptor substrate-1 (IRS-1), which inhibits insulin-stimulated tyrosine phosphorylation of IRS-1, leading to an increase in insulin resistance (3, 173). Considering the important role of the JNK pathway in the progression of DM, much effort has been made to suppress JNK activity in DM, and indeed, a cell-permeable JNK inhibitory peptide, derived from the JNK binding domain of JNK-interacting protein-1 (JIP-1), is effective for diabetes treatment (33).

In addition to the damage induced by elevated glucose, chronic exposure to increased plasma concentrations of FFA also leads to intramyocellular lipid accumulation in patients with T2DM, and this has been proposed to play a critical role in initiating and developing insulin resistance (31, 137). The increased metabolism of FFA causes ROS overproduction, which may contribute to the insulin resistance in muscle, adipocytes, pancreatic β-cells, and other cells (261).

3. DM complications. DM gives rise to many secondary complications, such as diabetic retinopathy, kidney disease, diabetic bone disorder, and heart diseases. The pathogenesis of these diseases is complex and is not well understood. However, recently some evidence has been proposed to show that the augmentation of ROS in the condition of DM may contribute to these complications. For example, AGEs are largely generated in DM, and the interaction between an AGE and its receptor (RAGE) generates oxidative stress and subsequently evokes vascular inflammation and thrombosis, both of which play important roles in the development of DM complications (343, 410). ROS-induced mitochondrial dysfunction and stress pathways involving PKC and JNK also contribute to this process. Mitochondrial dysfunction is a well-acknowledged risk factor for neurodegenerative diseases, and it is also one of the possible pathomechanisms for various complications that develop secondary to diabetes.

S-glutathionylation serves as a means for storing glutathione, and protects proteins form irreversible oxidation under oxidative stress (68), thus preventing the formation of abnormal networks regulating important events such as gene transcription and signal transduction (335). In DM and the related complications, reduced levels of glutathionylation appear to be associated with many key regulators of gene transcription and signal transduction, which may contribute to insulin resistance and hyperglycemia as well (242). For example, ROS are byproducts of the reaction catalyzed by aldose reductase (AR) which transforms cytosolic glucose into sorbitol, and increased glucose utilization by AR appears to be essential for the development of complications. A cysteine residue (Cys^{298}) is in the active site of AR, and S-glutathionylation of Cys^{298} in this active site can inhibit the activity of AR (345). It has been found that AR in DM complications is highly activated and that glutathionylation of AR is correspondingly decreased (242). Grx plays a key role in such regulation because it is a specific and efficient catalyst of deglutathionylation, and in DM complications, Grx is found to be upregulated, which may serve as a potent therapeutic target (333, 334).

E. Cancer

Higher levels of ROS are often observed in cancer cells as compared to normal cells. Also, ROS appear to play crucial roles in cancer development, through multiple signaling cascades related to various aspects of cancer cell biology, such as survival, proliferation, angiogenesis, and metastasis.

1. Role of ROS in cancer initiation. Over the past decades, studies have shown that intracellular redox state is crucial for carcinogenesis. For instance, mutagenesis studies using environmental factors such as ionizing radiation and xenobiotics, often result in the genesis of ROS in cells (49, 344). At the DNA level, ROS can directly target pyrimidines and purines, which eventually cause gene mutation. Also, ROS can target thiol-containing proteins to change the structure and activity of proteins. Thus, high levels of intracellular ROS target both DNA and proteins, and increase mutagenesis, convert protooncogenes into oncogenes, inactivate tumor-suppressor genes, and eventually result in differentiation block, uncontrolled cell growth, and impaired cell apoptosis (49, 107). The fact that dietary use of antioxidant food can decrease the probability of carcinogenesis may also represent a line of evidence for the critical role of redox reactions in tumorigenesis (47, 318).

Recently, ample evidence shows that the oncogenic process is closely related to the inflammatory response (14, 62). Professional phagocytes, including neutrophils and macrophages, exert their host defense response by killing invading microbes most likely through ROS production. However, in some conditions, ROS produced by the phagosome may also affect cells surrounding the lesion. In addition, by-products generated during inflammation such as HNE, malondialdehyde and other aldehydes can react with DNA bases or generate bifunctional intermediates, which are thought to be mutagenic and carcinogenic (74). Accordingly, it is deducible that altered redox reactions may represent a critical factor for cancer initiation.

2. Role of ROS in cancer cell proliferation. Cell proliferation requires activation of growth factor receptors and intracellular signaling pathways. Multiple lines of evidence have shown that oxidative stress can activate the ERK and PI3K/Akt pathways, which promote cancer cell survival under mild oxidative conditions (52, 104, 107). Redox modification can regulate ERK activation through phosphorylation by direct thiol-modification of the receptors, and through oxidative inactivation of phosphatases that dephosphorylate and inactivate receptors. Elevated ROS levels are also responsible for constitutive activation of transcription factors such as NF-κB (169) and AP-1 (385) that activate multiple genes involved in cell proliferation, during cancer initiation and progression.

3. Role of ROS in cancer cell metastasis. Metastasis occurs in a series of distinct steps that include tumor cell invasion, intravasation, extravasation, and proliferation. During these processes, moderate levels of ROS appear to be accompanied with and essential for the metastatic activity of cancer cells (98, 279, 353). Even during surgical procedures, a primary option for treating tumors, ROS production is correlated with the growth of metastatic cells, whereas treatment with antioxidants often inhibits the production of ROS and thus the growth of metastatic cells (149, 150).

Sustained MAPK signaling appears to be crucial in tumor metastasis, which relies on the cross talk between integrin, RTK, and PKC (405), and accompanied with the production of ROS. As a positive feedback, ROS participate in the promotion of signaling transduction, consequently resulting in cell adhesion, migration, and invasion (114, 263). Cytoskeleton rearrangement is also important for cell migratory, in which the Rho family of small GTPases (Rho, Rac1) act as critical regulators mediating extracellular and intracellular signals, and transferring them to effectors act on cytoskeleton (91). Strong evidence indicates that NOXs can be activated by Rac1, and the consequently generated ROS appear to downregulate the activity of Rho, showing a Rac-induced formation of membrane ruffles and integrin-mediated cell spreading (263). The role of ROS during this process is involved in oxidative inactivation of low-molecular-weight PTP, resulting in phosphorylation and activation of p190RhoGAP. Degradation of the extracellular matrix (ECM) is a critical step for cell invasion and metastasis. Invadopodia are actin-rich membrane protrusions found in invasive cancer cells, possibly exerting their role in degrading ECM. Previous studies indicate that one component of invadopodia, Tks5 (tyrosine kinase substrate with five Src homology 3 [SH3] domains), is a potent member of NOXs complex with a structure related to the Nox component $p47^{(phox)}$ and plays a role in ROS production and invadopodia formation (79). MMPs are the major enzymes responsible for the degradation of the ECM. Cancer cells with high metastatic potential have higher levels of MMPs as compared to other cancer cells with low metastatic potential (400). ROS have been reported to participate in regulating the redox-sensitive signaling pathway such as MAPK signaling cascades, to modulate MMPs expression and activity (144).

In addition, ROS produced by Rac1 after integrin engagement also contribute to the activation of some prosurvival signals involved in metastasis. For example, c-Src can be oxidized and activated by integrin-induced ROS, and activated c-Src can lead to EGFR activation in a ligand-independent manner and subsequently promote cell survival to anoikis (105). Some cancer cells with high metastatic potential showed a constitutive higher ROS production and a continuously oxidized/activated c-Src as compared to other less aggressive cancer cells (105).

One of the most important factors related to metastasis is HIF-1, a critical regulator of cell response to hypoxia. As mentioned above, expression of HIF-1α is largely dependent on intracellular O_2 concentration. Also, oncogene-dependent activation of HIF-1 in normoxia suggests that aerobic glycolysis for ATP generation is important in satisfying the needs of energy during the malignant progression (39, 187). Activation of HIF-1 leads to transcriptional activation of several glycolysis- and angiogenesis-related genes (230, 419). The role of HIF-1 in metabolic reprogramming, coupled with the finding that HIF-1 expression levels positively correlate with cancer invasiveness and poor prognosis, suggests that HIF-1 may be critical in linking tumor hypoxia, glycolysis energy metabolism, and cancer metastasis (258, 279). The decreased intercellular adhesion is a critical event during metastasis. Cadherins comprise a large family of transmembrane or membrane-associated glycoproteins that mediate specific cell–cell interactions in a Ca^{2+}-dependent manner (381).

Downregulation and inactivation of E-cadherin is the molecular hallmark of epithelial–mesenchymal transition (EMT), and contributes to the dedifferentiation and invasion of epithelial cells during tumorigenesis (288). Previous studies have indicated that the expression of E-cadherin is sensitive to the intracellular redox state and repressed by HIF-1 (45, 422).

Another interesting phenomenon regarding ROS as a causative agent in cancer metastasis is the involvement of mtDNA (153, 185). Using a technique of mtDNA exchange, one study group addressed the correlation between pathogenic-related mtDNA mutations and the metastatic potential during the malignant progression (154) (Fig. 9). mtDNA of an originally poorly metastatic mouse tumor cell line was replaced with mtDNA derived from a highly metastatic cell line, resulting in a significantly increased metastatic potential of the original cell line. It appeared that the transferred mtDNA contained mutations producing a deficiency in respiratory complex I activity, which was associated with ROS overproduction. Pretreatment of these metastatic cells with ROS scavengers suppressed their metastatic potential in mice (149, 182).

4. Aerobic glycolysis, an interesting metabolic mode for energy supply. In 1924 Warburg observed that some tumors preferentially utilized glycolysis instead of OXPHOS in the presence of O_2 (aerobic glycolysis). Since Warburg's pioneering studies, aerobic glycolysis has been reported in a wide variety of cancers. Normal cells prefer to use OXPHOS to produce more energy in normoxia, and glycolysis is a metabolic form for energy production when cells are deprived of O_2. However, cancer cells are more prone to glycolysis in normoxia for energy production, probably to support cancer cell growth rather than to compensate for defects in mitochondrial function, because most tumor cells have a substantial reserve capacity to produce ATP by OXPHOS when glycolysis is suppressed (95).

Cancer cells may benefit from aerobic glycolysis in the following respects. Aerobic glycolysis can decrease ROS production derived from the mitochondrial respiration chain during OXPHOS, and reduce the oxidative stress burden of cancer cells. In addition, the derivatives of glycolysis contribute to more than ATP generation during cancer cell proliferation. Glycolytic metabolites have been shown to be required for both fatty acid production and the maintenance of nonessential amino acid pools during growth. The generation of NADPH, a cofactor for many biosynthetic reactions, is produced in growing cells by oxidative degradation of glucose by glucose-6-phosphate dehydrogenase (G6PD). The resulting ribose is disposed through returning the carbon to the glycolytic pathway using the transaldolase/transketolase complex. Thus, although OXPHOS can produce more ATP than glycolysis, cancer cells prefer to use glycolysis to obtain energy since the glycolysis metabolic mode can supply ATP in a faster rate than OXPHOS, meeting the unlimited energy requirements during cancer progression.

5. Role of ROS in cancer cell chemosensitivity. Cancer has become the second highest cause of death in the world. Although significant progress has been made in cancer therapy during the past decades, most malignancies eventually relapse or become resistant to therapy. Drug resistance is a complex issue involving multiple interrelated or independent

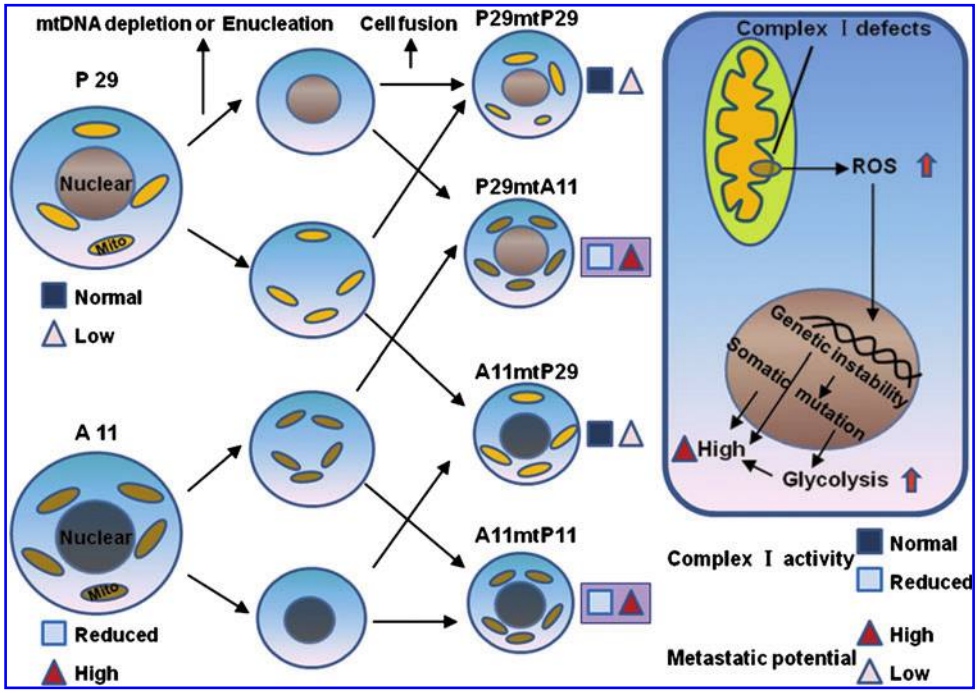

FIG. 9. ROS-generated mitochondrial DNA (mtDNA) mutation-regulated cancer cell metastatic potential. Ishikawa *et al.* applied mtDNA exchange technology to examine the involvement of pathogenic mtDNA mutations in malignant progression of tumor cells to develop metastatic potential. Two different cells were used. One was P29 cells with low metastatic potential and normal complex I activity, and another was A11 cells with high metastatic potential and complex I defects. Transfer mtDNA from the A11 cells to P29 cells makes P29 cells highly metastatic, and the metastatic potential of A11 cells was decreased when their mitochondria were replaced by the mitochondria from P29 cells. Defects of complex I-induced ROS overproduction, which may result in nuclear DNA mutation, metastatic-related gene expression, and adenosine triphosphate (ATP) depletion. The series events contribute to the enhanced glycolysis. P29mtP29 means P29 cells with its own mitochondria; P29mtA11 means P29 cells with A11 cells' mitochondria; A11mtP29 means A11 cells with P29 cells mitochondria; A11mtA11 means A11 with it own mitochondria [based on Ishikawa *et al*. (154)]; Mito, mitochondrion. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

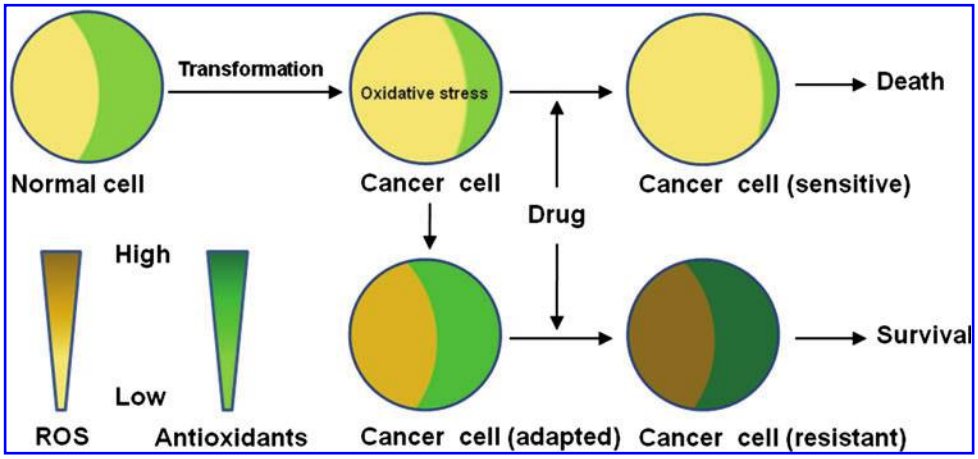

FIG. 10. Antioxidant state in relationship with chemosensitivity of cancer cells. Normal cells have a balance between oxidative species and antioxidants to maintain their physiological activity. Unlimited proliferation of cancer cells produces large amounts of ROS that induce oxidative stress. To balance the increased ROS, an adaptive response is achieved with the enhanced antioxidants system. Breaking this delicate balance by ROS-inducing drugs could induce cell death. Yet cells maintain the balance will be resistant to those drugs.

pathways, and reversal of chemoresistance in chemotherapy is a major problem in cancer therapy. Besides its positive role in cancer progression, ROS production is also a mechanism shared by most of nonsurgical therapeutic approaches. Several lines of evidence suggest that adaptation to oxidative stress and drug resistance may share common mechanisms (286). Tumor cells increase the amounts of ROS caused by the altered metabolic demand, which may induce the antioxidant adaptive response, and in turn, favors a redox imbalance, an altered redox regulation of cellular signaling, and the activation of prosurvival mechanisms (197) (Fig. 10). The antioxidant system is the front-line intracellular defense against oxidative stress. Cancer cells have different antioxidative systems for their adaption to a moderate level of intracellular ROS as compared to normal cells (286), and the enhanced ROS-scavenging systems partially participate in the chemoresistance process, through direct and indirect regulation of antiapoptotic and proproliferative genes to escape cytotoxic effects of the oxidative damage caused by chemotherapy drugs or radiation.

Intracellular concentration of GSH appears to be also an indicator for cancer cell chemosensitivity, as GSH is more abundant in some cancer cells than others, and is thought to be relevant to chemoresistance as well (197, 332). Given the role of GSH in cancer cell survival, several approaches designed to decrease intracellular GSH concentration have been pursued, including the use of buthionine-(S, R)-sulfoximine (BSO), a potent and specific inhibitor of γ-GCS, the rate-limiting step in the synthesis of GSH, as well as a hammerhead ribozyme against γ-GCS mRNA aiming specifically to downregulate GSH levels and to target c-Jun expression to reduce GSH levels (228). The combination therapy with GSH depletion agents and chemotherapy drugs has made promising headway *in vitro* and in murine models (11, 90, 197). In addition, the chemo/radiation resistant role of MnSOD has been recently implicated in human pancreatic or ovarian cancer cells (99, 413). TrxR, Gr/Grx, and Prx are important components in antioxidant system in the defensive mechanism against oxidative stress, and they are selectively upregulated in some cancer cells and probably involved in chemo/radiation resistance as well (197, 252).

Besides these nonenzymatic and enzymatic antioxidants, key transcription factors such as Nrf2, which play important roles in antiapoptotic processes under oxidative stress, cannot be ignored, as products of their target genes regulate many signal pathways, providing growth advantages to cancer cells (152, 198, 269).

6. Relationship between cellular redox state and CSCs. CSCs, with many characteristic features of normal stem cells, are tumorigenic (perhaps in contrast to other nontumorigenic cancer cells). These cells appear to persist in tumors as a distinct subpopulation and cause relapse and metastasis by giving rise to new lesions. Given their properties of long-term self-renewal, differentiation to downstream progenies, high repair capacity, and low sensitivity to xenobiotics such as chemotherapeutic agents, many scientists put forward the hypothesis that some of the major signaling pathways important in regulating normal stem cells may be applicable to CSCs as well.

Recent studies in CSCs have suggested that metabolic pathways in CSCs might be largely different from those in nontumorigenic cells, which may be responsible for maintaining stem cell features. In particular, ROS level has been found to be lower in some CSCs as compared to nontumorigenic cells, an observation consistent with the features of normal stem cells such as CNS stem cells or HSCs (80). Much evidence has indicated that the low level of ROS is important for maintaining the stemness features of stem cells. For instance, the interaction between HSCs and their microenvironment is important for maintaining normal hematopoiesis, and in bone marrow HSC enriched microenvironment (HSC niche) appears to be located at the lowest end of an O_2 gradient (282). Another example is that HSCs isolation based on their ROS levels in a competitive reconstitution assay, in which low ROS cells possess greater secondary and tertiary reconstitution capacity in lethally irradiated mice as compared to high ROS cells (160). These results underscore the significance of the environment, particularly hypoxia, in maintaining stem cell function.

The low level of oxidants is important for stem cells and in turn they develop a series of complex defense systems to

maintain their reduced state. Besides the upregulation of genes encoding antioxidants and antioxidative enzymes mentioned above, some transcription factors and signaling pathways responsible for stem cell differentiation are found to be redox-sensitive as well. For example, Oct4, an important transcriptional factor for maintaining stem cell totipotency, binds directly to the antioxidant Trx, which has a role in protecting Oct4 from oxidizing agents (115).

To date, knowledge about CSCs is primarily based on what we have learnt from embryonic stem cells (ESCs) or normal HSCs. As the isolation of CSCs still represents a major stumbling block in cancer researches, studies of CSCs lag behind. However, using a Hochest 33342 dye perfusion method has been successful to some extent in isolating CSCs in leukemia and many solid tumors (355). Accordingly, by measuring ROS levels to isolate CSCs may represent another practical approach when we have a good knowledge of the redox status of CSCs.

V. Perspectives in Clinical Treatment

ROS are involved in a variety of diseases and thus therapeutic approaches using antioxidants are widely investigated. Some of these approaches appear to be successful, whereas many others have little or no benefit in disease prevention or treatment. Traditional antioxidants such as vitamin C and E always exert their antioxidant ability in a nonspecific and hysteretic manner, and the major intent is to mitigate the effects of ROS that already have been produced. Currently, delivery of antioxidants to the target location in the right time frame is being widely studied.

A. Pharmacological targeting of mitochondria

Mitochondria are responsible for intracellular energy production and play a central role in cell survival and death. As reviewed here, a wide range of diseases, including diabetes, neurodegenerative diseases, cardiovascular diseases, and cancer, are associated with mitochondrial dysfunction, and pharmacological targeting of mitochondria in these diseases has been widely studied.

Approaches with mitochondria-targeted drugs mainly focus on the ETC and mitochondrial membrane dynamics. For instance, complex III is one of the major sites responsible for ROS generation in ETC as well as an O_2-sensor in hypoxia through regulating HIF-1α (24). In addition, complex III plays a key role in regulating glutathione-dependent mitochondrial membrane permeabilization and apoptosis, thus representing an ideal target in cancer treatment (16). Some mitochondrial membrane shaping proteins have also been identified in the regulation of apoptosis through modulating cytochrome c release, providing a potential role as novel targets for death regulation in cancer.

It has been known for many decades that antioxidants have a protective role against oxidative stress-induced injury in clinical settings. Therefore, delivery of antioxidant drugs to dysfunctional mitochondria with minimal side effects to other organelles has attracted much attention for the treatment of many diseases. Due to the unique features of mitochondria, namely, their transmembrane potential and their organelle protein import machinery, the application of mitochondria-targeting delivery systems appears to be a feasible approach (15, 409). In the following part, we give a brief description of three mitochondria delivering vehicles: mitoquinone (MitoQ), dequalinium liposomes (DQAsomes), and Szeto-Schiller (SS) peptides.

MitoQ is a novel oral antioxidant that covalently binds with coenzyme Q (coQ10). Compared to nontargeted coenzyme Q (CoQ10) analog decylubiquinone, which shows a deficiency in phosphonium ion, MitoQ can cause a several hundred folds enrichment in mitochondria, thus protecting cells against oxidative damage (159). MitoQ has been shown to be potently protective in an *ex vivo* model of IR injury (2, 15). The latest study has indicated that administration of MitoQ for 12 weeks can effectively prevent diabetic nephropathy in mice (50). Currently, MitoQ is being evaluated in clinical trials in PD (341)

Another category of effective mitochondria-targeting delivery systems is represented by DQAsomes. These liposomes appear to be efficiently taken up by endocytosis and quickly fused to the outer membrane, allowing mitochondria signal peptide (MSP)-tagged cargo to enter the matrix *via* the protein import system (396, 397). This system can limit the direct exposure of the drug to P-glycoprotein multidrug efflux pump and may overcome drug resistance.

Besides the two categories of the delivery systems described above, SS peptides constitute another class of mitochondria delivering vehicles. The sequence motif of these peptides can target them to mitochondria in an energy independent and nonsaturable manner. SS peptides are rapidly taken up into cells and reach a steady-state concentration within minutes with minimal toxicity (15, 354). The structural motif of these SS peptides centers on alternating aromatic residues and basic amino acids (aromatic-cationic peptides) (354, 423). Their antioxidant action can be attributed to the tyrosine or dimethyltyrosine (Dmt) residue. Tyrosine can scavenge oxyradicals forming relatively unreactive tyrosyl radicals, which can be followed by radical–radical coupling to give dityrosine, or react with superoxide to form tyrosine hydroperoxide (403). Based on these characteristics, SS peptides can be used as delivery devices for death inhibitors or apoptosis activating drugs. Recently, SS peptides have been shown to protect against IR-mediated tissue injury and neurodegenerative disease in animal models (59).Taken together, pharmacological targets of mitochondria in disease mainly focus on the ETC, mitochondrial membrane dynamics and mitochondria-directed delivery systems. These mitochondria or oxidative-stress targeting approaches have shown promising potential in the treatment of a wide range of diseases discussed in this review.

B. Pharmacological targeting of NOXs

Since the discovery of ROS generated by NOXs in nonphagocytes, the participation of NOXs as an important, and in many cases the predominant, source of ROS in various diseases is greatly appreciated. With advances in NOXs studies, increasing evidence has indicated that NOXs are promising pharmacologic targets for oxidative-stress-induced diseases (161). As aforementioned, Rac GTPases are important activators of NOXs in regulating ROS generation, and many studies suggest that expressing dominant-negative protein of Rac subunit can inhibit ROS generation (181, 303). In addition, the search for NOXs inhibitors has identified many promising candidates derived both from nature and from chemical

synthesis. In the following part, we give a brief description of two commonly used NOXs inhibitors.

Diphenylene iodonium (DPI), which is the most widely used inhibitor of NOXs, can inhibit all NOXs and DUOXs isoforms at micromolar to submicromolar concentrations. Their inhibitory effect occurs through acceptance of an electron from the flavin to generate a radical species that then covalently modifies the flavin coenzyme itself or its active site (193). DPI is an effective inhibitor of NOXs *in vitro*, but is unsuitable as a candidate for clinical use because of its side effects. It is reported that the effective concentration of DPI for NOXs can also inhibit the NADPH-coenzyme Q reductase and nitric oxide synthase, and other flavoprotein dehydrogenases can also be affected (349). Hypoglycemia is an acute adverse effect induced by DPI (7). Despite that, DPI has made a great contribution to the functional characterization of NOXs.

Apocynin is a natural methoxy-substituted catechol isolated from *Picrorhiza kurroa* that has been used as an inhibitor of NOXs both in phagocytes and in other cell types since the early 1990s. Despite some controversial results of apocynin in nonphagocyte cells, consistent results in phagocytes have confirmed its role in inhibiting NOXs, which is probably due to the presence of MPO, since apocynin needs to be converted to a dimer in a reaction that involves ROS-dependent cross-linking by one peroxidase to exert its inhibitory effects on NOXs (407). The pharmacological mechanism of apocynin in nonphagocytic cells is still unclear. In animal models with pathologies such as ischemia-reperfusion injury or hypertension, apocynin exhibits high potency in suppressing the symptoms after short- or long-term administration (290, 411). The debate concerning apocynin as an inhibitor of NOXs has emerged from recent studies which indicate that in nonphagocytic cells, apocynin functions as more like an antioxidant rather than an inhibitor of NOXs (133). As a radical scavenger, apocynin is active at a concentration above 100 μM, but in neutrophils the IC_{50} of apocynin to inhibit NOX enzymes is 10 μM (378).

In conclusion, through many years of efforts, a large number of NOX inhibitors have been made available (161). However, there is still much challenge in their utilization in clinical settings because of incomplete characterization of their pharmacological properties and their unforeseen side effects. The side effects of long-term administration of NOX inhibitors are still under study. One possible side effect of NOX inhibition may be the suppression on phagocyte innate immune response. Thus, developments using the inhibitors directly targeting NOXs' isoenzymes with a high specificity may overcome some side effects and even resolve some of the problems posed by antioxidants. Researches on peptide-based NOX inhibitors may provide a promising direction for the future. For example, one team developed a peptide inhibitor based on B loop of NOX2, the mutation of which disrupts the assembly of NOX2 with cytosolic regulatory proteins (308). In rat models of hypertension and atherosclerosis, infusion of this peptide was effective in inhibiting Ang II-induced superoxide production and hypertension (156, 164).

C. Targeting XO-derived ROS

As mentioned above, XO-derived ROS are involved in vascular disorders and participate in a variety of diseases, including ischemia-reperfusion injury and atherosclerosis. Under normal conditions, XOR exists in the form of XDH, and a high concentration of NAD^+ inhibits generation of ROS by XDH. However, in some pathological conditions, especially in ischemia-reperfusion injury, it has been proposed that ROS produced by XO are increased by the accelerated conversion of XDH to XO, the accumulated ATP degradation products, including hypoxanthine and xanthine, and the upregulated XDH/XO mRNA (34) (Fig. 11). In addition, it has been observed that XO is released into the circulation from damaged organs, which promotes ROS generation at sites distant from the initial site of XDH/XO release and accelerates pathological changes (302). Circulating XO binds with high affinity to endothelial cells through electrostatic interactions with glycosaminoglycans in a partially heparin-reversible manner (302). Upon immobilization, XO becomes resistant to inactivation and acquires modified kinetic characteristics. Initially, it was considered that circulating XO could be used as a sensitive marker of liver injury. Subsequent works demonstrated that circulating XO is not only a marker of hepatic and intestinal damage, but is also responsible for remote organ injury in a variety of pathophysiological conditions, including hepatic ischemia and reperfusion, hemorrhagic shock, atherosclerosis, and sickle cell disease (358, 359). However, currently, the role of XDH/XO in the pathogenesis of these injuries is still unclear.

Although it is unclear whether XO inhibitors will have a positive effect on the therapy of diseases mentioned above, some XO inhibitors such as Allopurinol play protective roles in these injuries (34, 277). Allopurinol, which was initially synthesized in the mid-1950s by Falco in an attempt to produce new antineoplastic agents, was found to be an effective

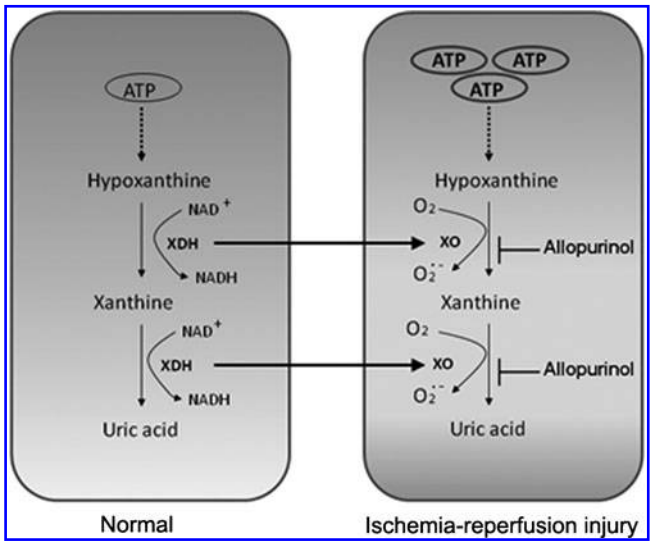

FIG. 11. Inhibitory role of Allopurinol in XO-induced ROS during ischemic-reperfusion. Under normal conditions, XDH is the major form of xanthine oxidoreductase (XOR) and is responsible for catalysis of ATP degradation products (hypoxanthine, xanthine) in a NAD-dependent manner. In ischemic-reperfusion conditions, ROS are overproduced by the accelerated irreversible conversion of XDH to XO and the increased ATP consumption. Allopurinol can bind to the active site of XO to inhibit its activity to decrease the production of ROS.

XO inhibitor. Allopurinol is a substrate of XO and competitively binds to the active site of XO to inhibit XO activity (Fig. 11).

In the clinical setting, Allopurinol is mainly used in the treatment of gout, which is a form of inflammatory arthritis, associated with hyperuricaemia, in which the formation of monosodium urate crystals in the joints and periarticular tissues causes acute inflammatory attacks as well as long-term tissue damage. It has also been found that Allopurinol administration decreases tissue injury after ischemia/reperfusion in a variety of *in vitro* and *in vivo* models (356). Although it is generally believed that this protective effect is due primarily to the inhibitory effect on XO activity, some investigators have suggested that Allopurinol may also act as a nonspecific scavenger of ROS at much higher concentrations than when used as an XO inhibitor (321).

D. ROS-generating agents as cell death inducers

Cancer cells have been demonstrated to have a universal glycolytic phenotype. Shifting the cellular redox balance through pharmacologic manipulation in favor of increasing intracellular ROS may lead to oxidative stress and subsequently induce apoptosis within cancer cells. Arsenic trioxide (ATO) has significant effects on the treatment of acute promyelocytic leukemia (APL), which make ROS-generating agents that induce oxidative stress a favorable therapeutic option. Here we will highlight two important agents, ATO and fenretinide.

1. Arsenic trioxide. Arsenic, as a semimetal, is well known for its carcinogenic effects, whereas the inorganic form of arsenic, ATO, has been widely accepted for its antitumor activity (294). It has been successfully used in APL treatment for many years. Treatment with ATO can induce the degradation of PML-RARα fusion protein and induce leukemia cell differentiation/apoptosis (190). The PML-RARα fusion protein has been found in the vast majority of cases of APL, and is the gene product of the chromosomal translocation t(15;17) (86). This fusion protein behaves as a transcriptional repressor that suppresses the expression of genes essential in normal myeloid differentiation. The behavior of ATO is influenced by its concentration. A low concentration of ATO in APL induces leukemia cells to differentiate, whereas a high concentration of ATO can trigger cell apoptosis (53). Since this initial observation, numerous studies have focused on ATO in anticancer activity in various types of cancer. ATO has been shown to stimulate PCD in neuroblastoma, multiple myeloma, and other hematopoietic cell lines (291, 312). Mechanisms involved in apoptotic effects of ATO are complex and not fully understood. However, much evidence has indicated that arsenic can disturb natural oxidation–reduction equilibria to induce oxidative stress in cancer cells (245, 246, 350). Arsenic is a semimetal, and like other metals, it has the ability to induce the generation of free radical species. Although the pathway responsible for arsenic-induced ROS generation remains unknown, it has been reported that exposure to arsenicals stimulates ROS generation in cultured cells through NOXs, mitochondrial ETC, and the inhibition of antioxidant enzymes such as GPx and TrxR (123, 124, 391).

ATO has been reported to show a high binding affinity to proteins containing vicinal SH groups (245). SH groups exist in many protein structures, especially in the active sites of many enzymes. The binding of ATO may destroy the redox-regulation of these enzymes. In addition, it has been reported that the oxidative damage induced by ATO can be attributed to the dysfunction of mitochondria through destruction of the mitochondrial inner transmembrane potential to promote apoptosis (43). Experiments with purified mitochondria show that administration of ATO promotes the opening of the permeability transition pore, releasing intermembrane proteins, which ultimately causes caspase activation.

ATO has been shown to induce apoptosis in many types of malignant cells, although their sensitivities to ATO are different. Certain mediators responsible for the sensitivity of ATO have been identified. NB4 cells, derived from APL, can be rendered apoptotic by ATO at a relatively low concentration. However, it has been found that some ATO-resistant NB4 subclones also exist. Mechanisms responsible for the different sensitivity to ATO may be complex and related to multiple factors. However, much evidence has indicated that the intracellular GSH redox system may contribute, at least in part, to this differential sensitivity (66, 166). ATO-resistant NB4 cells contain relatively low levels of glutathione peroxidase and CAT and higher levels of intracellular H_2O_2. Administration of BSO, which diminishes GSH levels, promptly restores the sensitivity of these cells to ATO. Other studies have found that a relatively lower GSH redox system existed in other malignant cells sensitive to ATO (66). Treatment with some agents that can scavenge intracellular ROS and enhance the activity of the GPx (*e.g.,* selenite) can decrease the sensitivity to ATO.

Combining ATO with some other anticancer agents may enhance therapeutic effects, as typically highlighted by its combination with all-*trans* retinoic acid (ATRA) (425, 426). ATRA, as the first-line drug in the treatment of APL, primarily acts through the release of the repressive effects of the PML-RARα fusion protein to induce APL cell differentiation into matured neutrophils (415). ATO has been proposed as an alternative to treatment with ATRA because it can induce complete remissions in both RA-sensitive and RA-resistant APL patients, and is also effective in relapsed APL patients treated with ATRA (53, 342). Combination treatment with ATO and ATRA exhibits favorable effects in animal models and in clinical studies of APL. In a xenograft mouse model of APL, treatment with either ATO or ATRA increased survival by 35%–39%. However, when these mice were treated sequentially with either ATO or ATRA followed by the other agent, their survival was prolonged by 70%–80% (167). In clinical setting, the combination of low dose ATRA and ATO also provided significantly better therapeutic effects (390). In addition, combination treatments of ATO and other chemotherapy drugs have also exhibited a notable effect in many types of cancer. For example, ATO combined with Imatinib mesylate (STI571) has shown synergistic effects on chronic myeloid leukemia (CML) cells. STI571 is a specifically designed inhibitor that targets the tyrosine kinase activity of the BCR-ABL fusion protein and it consequently induces CML cells apoptosis *in vitro* as well as *in vivo* (238, 322). However, a significant proportion of the treated patients who previously failed to respond to interferon therapy remained predominantly BCR-ABL, suggesting a risk for later relapse (347). Further, patients in the accelerated and blast-crisis phase revealed a high frequency of relapse or resistance to STI571

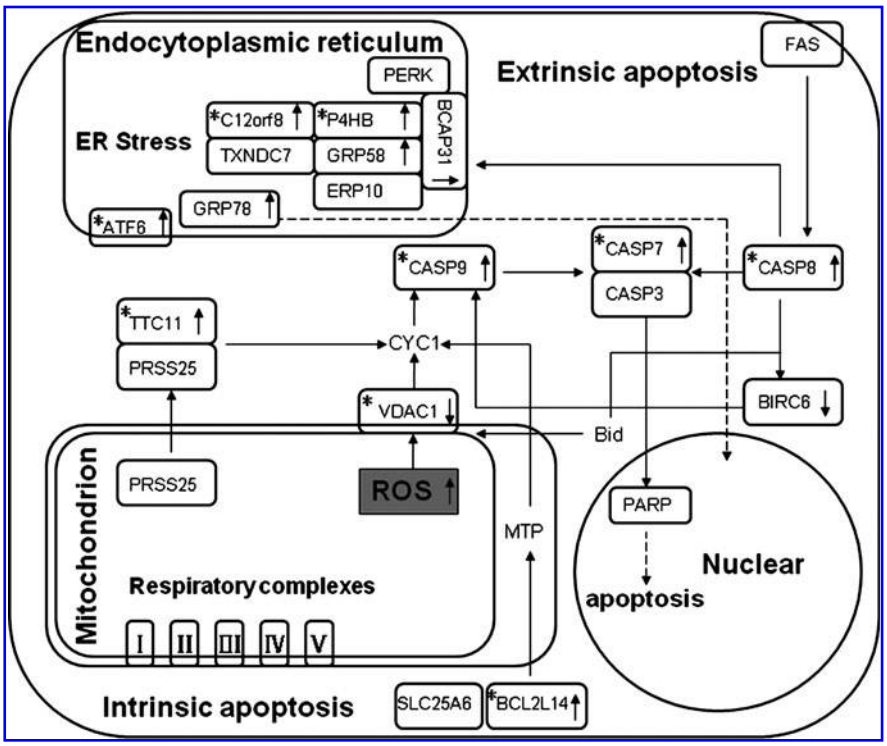

FIG. 12. Ideogram illustration of intrinsic, extrinsic, and ER stress pathways involved in STI571/arsenic trioxide (ATO)-induced apoptosis in chronic myeloid leukemia (CML). Genes especially involved in apoptosis pathway modulated in STI571, ATO, and the cotreatment of STI571 and ATO are shown by *up-arrows* (upregulation) and *down-arrows* (downregulation); *synergistically/additively affected genes in the cotreatment series. [The detailed information can be obtained from Du *et al.* (85).]

(135). As a result, much interest is focused on the development of combination therapies to improve response rates and prevent resistance or relapse. The combinatory treatment of ATO and STI571 has shown a promising potential in resolving this problem. STI571 can inhibit BCR-ABL tyrosine kinase activity and ATO can lower BCR-ABL levels (297). Previous work by our group has depicted dynamic changes underlying proapoptotic and apoptotic activities occurring after drug exposure of the CML-derivative K562 cells (85). We found that although oxidative stress resulting from a subtoxic concentration (1 μM) of ATO alone was insufficient to induce cell apoptosis in K562 cells within the observation time, its induced ER stress appeared to contribute significantly to the synergistic induction of apoptosis in the cells cotreated with ATO and STI571. Enhanced apoptotic activities are further suggested by addition of cascades of the ER stress-mediated pathway and probably the extrinsic apoptotic pathway as well. Coordinated regulation of the genes/proteins relevant to these pathways may consequently result in an effective and efficient induction of cell apoptosis in K562 cells treated with STI571 combined with ATO (Fig. 12).

2. Fenretinide. Recently, new therapeutic intervention strategies producing a state of selective oxidative stress in cancer cells have gained importance. There is considerable interest in designing the most rational redox-active strategies with minimal *in vivo* side effects. In this respect, fenretinide, that is, N-(4-hydroxyphenyl)retinamide (4HPR), a synthetic retinoid tested in several long-term clinical trials, is worthy of further investigation. Fenretinide was first produced by R.W. Johnson Pharmaceuticals in the late 1960s and has been known to be effective in cancer chemoprevention (373). The chemopreventive role of fenretinide against carcinogenesis of the breast, prostate, and skin has been demonstrated in animal models. Moreover, in clinical use, fenretinide protects against the development of a secondary breast malignancy in premenopausal women who had been treated to prevent the progression of early-stage breast cancer, and prevents relapse and the formation of secondary primary lesions in patients after the surgical removal of oral leukoplakia (56, 384).

Unlike natural retinoids such as ATRA, fenretinide induces distinct biologic effects, including apoptosis, in many tumor cells through generation of ROS and lipid second messengers while maintaining its minimal *in vivo* cytotoxicity to normal cells (118). How fenretinide induces apoptosis has been explored intensively. Apoptosis induced by fenretinide can be inhibited by antioxidants like vitamin C, N-acetylcysteine, butylated hydroxyanisole, and pyrrolidine dithiocarbamate, which suggests an essential role of ROS in the process (13, 110). It is also reported that the increase of ceramide levels is accompanied by the apoptotic process (237, 307).

Recently, much evidence has indicated that the engagement of ROS in cancer cells apoptosis induced by fenretinide is probably accompanied by the activation of ER stress (13, 170), which has also demonstrated the symbiotic relationship between oxidative stress and ER stress in some conditions. To uncover regulatory mechanisms underlying the conversion from oxidative signaling to downstream stress events exerted on ER and eventually to cell death outcomes rather than survival advantages, we employed integrative methods of advanced data mining with microarray technology to profile transcriptome changes in a fenretinide-sensitive cell line, and found numerous temporal-spatial relationships between stress-responsive events (392) (Fig. 13). Moreover, Nrf2 and heat-shock transcription factor 1 (HSF1), two critical stress-responsive transcription factors, appear to play prominent roles in the configuration of these relevant events. Experimental evidence suggested that activation of Nrf2 and thus its

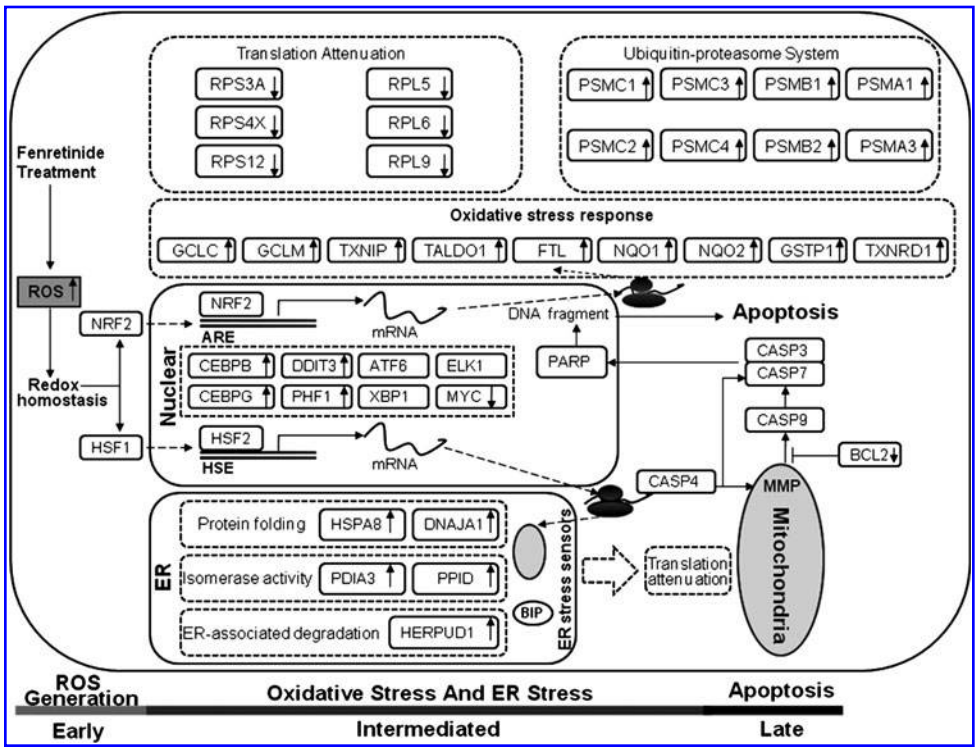

FIG. 13. Ideogram illustrating temporal-spatial relationships among major stress-responsive events relevant to oxidative stress-mediated apoptosis in NB4 cells. Early, intermediate, and late stage apoptosis and their characteristic events are indicated on the bar underneath. Genes/proteins upregulated are marked by *up-arrows*, and those downregulated are marked by *down-arrows*. [The detailed information can be obtained from Wang *et al.* (392).]

target genes may contribute to reduction of ROS levels, as observed during the intermediate and late stage, whereas activation of HSF1 and thus its target genes may contribute to the transient occurrence of UPR. We speculate that although these two sets of stress-responsive genes are individually considered as regulators of cellular defense mechanisms, their coordinated regulation in such manner as consistent activation of Nrf2 targets and transient activation of HSF1 targets can be critical for the effective progression of apoptosis in response to fenretinide stimuli. Although detailed relationships between UPR and subsequent cell apoptosis remain to be clarified, UPR termination before the late stage is probably essential for the effective activation of apoptosis (392).

E. Application of systems biology in biomedical researches

Three scientific breakthroughs have markedly accelerated our understanding and treatment of human diseases over the past half-century. The first is the progressive elucidation of the genetic basis of biological information (394), from information storage (DNA) to processing (RNA) and to execution (proteins and metabolites). The second is the evolution of high-throughput omics technologies (92), including genomics, transcriptomics, proteomics, and metabolomics, which quantify the extent of change in various genome-wide biological information in a simultaneous, parallel, and automated manner. The third consists of various conceptual advances in systems biology (271), allowing the integration of disparate omics data into a network-like understanding of the underlying pathogenesis of human diseases. These advances hold great promise for the identification and characterization of potential drugs, their modes of action, and their molecular targets, with the ultimate goal of predictive, preventive, and personalized medicine (138). Although to achieve such predictive, preventive, and personalized medicine may represent a distant goal, it has now become feasible to address complex questions using omics approaches, since they usually do not fall into the category for general molecular biology questions. For example, the observation that ATO significantly potentiates the effect of imatinib for apoptosis induction in CML cells typically represents a complex question, and accordingly we have first applied a transcriptome approach to profile CML cells in time series, respectively, treated with ATO, imatinib, and the combination of the two. Through a robust tool of data analysis and visualization (*i.e.*, component plane presentation–self-organizing map [CPP-SOM]), and followed by validations using methods of molecular biology and protein biochemistry, we have shown that imatinib primarily triggers the intrinsic pathway of cell apoptosis, whereas ATO induces the ER stress-mediated cell apoptosis, and the combination of the two agents appears to more effectively induce the intrinsic, extrinsic, and ER stress-mediated pathways of cell apoptosis, which results in a more effective and efficient induction of PCD in CML cells (85).

Another relevant example is the systems analysis of fenretinide-induced apoptosis (392), in which we have applied a similar approach but significantly modified our tools of data mining. Briefly, gene transcriptome data are first subjected to robust gene selection that integrates SOM for data preprocessing and singular value decomposition (SVD) for pattern recognition (8). Using multiple testing procedures for false-discovery rate estimation, our hybrid SOM-SVD bases the entire gene selection process on statistical inference, allowing the maximum retention of information inherent in the primary microarray data. For gene clustering and visualization, the selected gene expression data are analyzed by CPP-integrated SOM (406). Essentially through such a

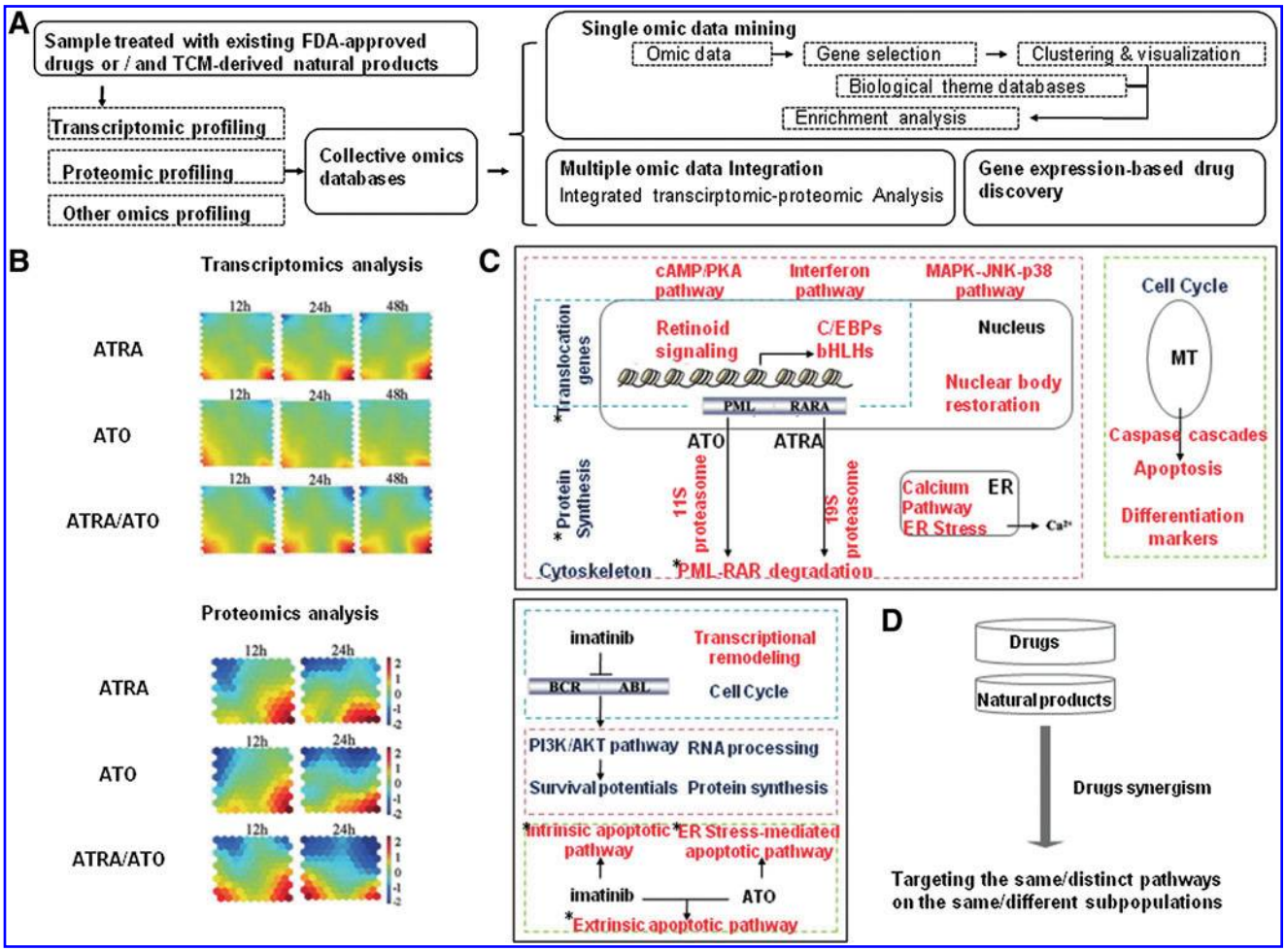

FIG. 14. Transcriptome and proteome analysis process of the synergism between drugs and/or natural products. (A) Framework of high-throughput omics technologies in achieving effective and efficient drug development. Cultured human cancer cells are exposed to a single agent or a combination of agents (drugs and/or natural products). The treated samples are subjected to transcriptomic, proteomic, and other omics analyses in a simultaneous, parallel, automated manner. Gene expression profiles are submitted to collective omics databases. Data mining processes ranging from single omic data mining (*e.g.,* transcriptomic data analysis procedures, such as gene selection, gene clustering, and visualization, and enrichment analysis of biological themes), to multiple omics data integration (*e.g.,* integrated transcriptomic-proteomic analyses), and GE-HTS and CMap for drug discovery. **(B)** Component plane presentation–self-organizing map (CPP-SOM) illustrates the dynamic changes in the transcriptome and proteome of NB4 leukemia cells treated with all-trans retinoic acid (ATRA), ATO, or ATRA/ATO. Each map presentation illustrates treatment-specific transcriptome (or proteome) changes, in which up- (red), down- (blue), and moderately regulated (yellow and green) genes are well delineated. The color bar represents the expression level (log ratio with base 2). **(C)** Ideogram illustrating the temporal-spatial relationships among major molecular events occurring during ATRA/ATO-induced differentiation/apoptosis in acute promyelocytic leukemia (APL) (*top panel*) or during imatinib(STI571)/ATO-induced apoptosis in CML (*down panel*). Molecular events enriched with upregulated genes/proteins are marked in red, whereas those enriched with downregulated genes/proteins are marked in blue. Synergistically regulated events are highlighted with asterisks. Events occurring at the early, intermediate, and late stages are outlined by cyan, pink, and green dotted lines respectively. Also indicated as necessary are the intracellular compartments, including the nucleus, ER, and mitochondria (MT). **(D)** Synergistic therapeutic actions of drug/natural product combinations. Drug synergism can be achieved by targeting the same pathway or distinct pathways through the integrative approach mentioned in **(A)**, **(B)**, and **(C)**. [The detailed information can be obtained from Fang *et al*. (94).]

transcriptome approach, a network-like description of fenretinide-induced apoptosis in leukemic calls has been obtained, highlighting tempo-spatial changes of important events, such as Nrf2- or HSF1-mediated gene transcription, ROS and UPR, triggered by fenretinide. Although such a description may not fit the scope of general molecular biology, it provides a rough roadmap to quickly understand fenretinide-induced apoptosis and probably to facilitate many aspects of fenretinide-involved translational research as well. For instance, the proteasome system appears to be strongly activated during the fenretinide-induced apoptosis, and the addition of a proteasome inhibitor has indeed potentiated the power of fenrentinide to induce apoptosis in cancer cells (392). Figure 14 summarizes information about our analysis of ATRA/ATO-induced differentiation/apoptosis in APL and imatinib/ATO-induced apoptosis in CML (85, 94, 425).

VI. Conclusion

In conclusion, ROS are multifaceted regulators essential for many normal physiological processes. However, when chronically present or transiently generated at non-physiologically high levels, ROS initiate various pathological states leading to a wide range of diseases. Based on such a double-edged-sword nature, a variety of approaches and agents have been developed to intervene with ROS levels in disease cells. Due to the diverse physiological functions of ROS in cells, nevertheless, a comprehensive understanding of mechanisms underlying ROS-mediated activities in normal or/and disease cells is required to develop more sophisticated protocols for the treatment of diseases. In this regard, system biology may provide the necessary tools. Various techniques of omics allow the quantitative measurement of biological molecules (*e.g.,* genes and proteins) and their modifications (*e.g.,* methylation and demethylation, acetylation, and deacetylation) at the genome-wide scale, and advanced computational tools permit the integration of information from multiple layers, such as at the layers of transcriptomics, proteomics, metabolomics, genomics, and epigenomics. It is predictable that using therapy with advanced ROS modulation to cure many of the above diseases in near future will come true.

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Abbreviations Used

4HPR = N-(4-hydroxyphenyl)retinamide
8-OHdG = 8-hydroxy-2′-deoxyguanosine
8oxodG = 7, 8-dihydro-8-oxoguanine
γ-GCS = γ-glutamyl cysteine synthetase
$\Delta\psi_m$ = mitochondrial membrane potential
Aβ = amyloid-β peptide
ABAD = Aβ-binding alcohol dehydrogenase
AD = Alzheimer's disease
AGE = advanced glycation end-products
AGT = angiotensin II
ALDH4 = aldehyde dehydrogenase 4
ALS = amyotrophic lateral sclerosis
APL = acute promyelocytic leukemia
apoB100 = apolipoprotein B-100
APP = amyloid precursor protein
AR = aldose reductase
ARE = antioxidant-responsive element
Atg = autophagy-related genes
ATO = arsenic trioxide
ATP = adenosine triphosphate
ATRA = all-trans retinoic acid
BSO = buthionine-(S, R)-sulfoximine
bZIP = basic leucine zipper proteins
CAT = catalase
cGMP = cyclic guanosine monophosphate
CML = chronic myeloid leukemia
CMVEC = cerebral microvascular endothelial cells
CNS = central nervous system
CPP = component plane presentation
CSCs = cancer stem cells
CYP = cytochrome P450
CyPA = cyclophilin A
Cys-SOH = catalytic sites to sulfenic acids
DM = diabetes mellitus
Dmt = dimethyltyrosine
DPI = diphenylene iodonium
DQAsomes = dequalinium liposomes
Dvl = dishevelled
ECM = extracellular matrix
EGF = epithelial growth factor
eNOS = endothelial nitric oxide synthase
EMT = epithelial–mesenchymal transition
ER = endoplasmic reticulum
ERK = extracellular signal-regulated kinase
ERO1 = endoplasmic reticulum oxidoreductin 1
ETC = electron transport chain
FAD = flavin adenine nucleotide
FALS = familial amyotrophic lateral sclerosis
FFA = free fatty acids
FIH-1 = factor inhibiting HIF
Foxo-1 = forkhead box O-1
FZD = Frizzled
G6PD = glucose-6-phosphate dehydrogenase
GLS2 = glutaminase
Gr = glutathione reductase
Grx = glutaredoxins
GSH = L-γ-glutamyl-L-cysteinyl glycine (glutathione)
GSH-Px = glutathione peroxidase
GSH-Px-1 = glutathione peroxidase-1
GSSG = glutathione disulfide
H_2O = water
H_2O_2 = hydrogen peroxide

Abbreviations Used (Cont.)

HD = Huntington's disease
HDAC = histone deacetylase
HIF-1 = hypoxia inducible factor 1
HNE = 4-hydroxynonenal
HO• = hydroxyl radical
HRE = hypoxia-responsive element
HSCs = hematopoietic stem cells
HSF1 = heat-shock transcription factor 1
ICAM-1 = intercellular adhesion molecule-1
iNOS = inducible form of nitric oxide synthase
INrf2 = inhibitor of Nrf2
IRS-1 = insulin receptor substrate-1
JIP-1 = JNK-interacting protein-1
JNK = c-Jun N-terminal kinase
JunD = jun D proto-oncogene
KEAP1 = Kelch-like ECH-associated protein 1
LDL = low-density lipoprotein
LPO = lipid peroxidation
MafA = v-maf musculoaponeurotic fibrosarcoma oncogene homolog A
MAPK = mitogen-activated protein kinase
MAP2K = MAP kinase kinase
MAP3K = MAP kinase kinase kinase
MEF = mouse embryonic fibroblast
MMPs = matrix metalloproteinases
MnSOD = manganese superoxide dismutase
MPO = myeloperoxidase
MPTP = 1-methyl 4-phenyl-1, 2, 3, 6-tetrahydropyridine
MSP = mitochondria signal peptide
mtDNA = mitochondrial DNA
NF-κB = nuclear factor-kappa B
NOXs = NADPH oxidases
Nrfs = nuclear factor (erythroid-derived 2)-related factors
Nrx = nucleoredoxin
$O_2\bullet^-$ = superoxide
ODD = O_2-dependent degradation
OXPHOS = oxidative phosphorylation
p66ShcA = p66 isoform of the ShcA adaptor protein
PARP-1 = poly(ADP-ribose) polymerase-1
PC = phosphatidylcholine
PCD = programmed cell death
PD = Parkinson's disease
PDGF = platelet-derived growth factor
PDI = protein disulfide isomerase
PDX1 = pancreatic and duodenal homeobox 1
PERK = PRKR-like endoplasmic reticulum kinase
PGC = peroxisome proliferator-activated receptor gamma
PHD = prolyl hydroxylase domain
PI3K = phosphoinositide 3-kinase
PIG3 = P53-induced gene 3
PINK1 = phosphatase and tensin homologue (PTEN)-induced kinase 1
PIP3 = phosphatidyl-inositol 3,4,5-triphosphate
PKC = protein kinase C
protein-SSG = protein-glutathione mixed disulfide
Prxs = peroxiredoxins
PTEN = Phosphatase and tensin homolog
PTP1B = protein tyrosine phosphatase, nonreceptor type 1
PTPases = protein tyrosine phosphatases
PTPs = protein tyrosine phosphatases
PUFA = polyunsaturated fatty acid
PUMA = p53 upregulated modulator of apoptosis
RAGE = advanced glycation end-product receptor
RCS = reactive carbonyl species
RET = reverse electron transfer
RIP1 = receptor interacting protein -1
ROS = reactive oxygen species
RTK = receptor tyrosine kinases
SAPKs = stress-activated protein kinases
SH = sulfhydryl
Sirt1 = sirtuin 1
SOD = superoxide dismutase
SOM = self-organizing map
SS = Szeto–Schiller
STI571 = Imatinib mesylate
SVD = singular value decomposition
TCF = T cell factor
TGF-β = transforming growth factor β
TNF-α = tumor necrosis factor-α
TNFR1 = TNF receptor 1
TPA = 12-O-tetradecanoylphorbol-13-acetate
TRADD = TNFR1-associated death domain protein
TRAF2 = TNF receptor-associated factor -2
TRAIL = TNF-related apoptosis-inducing ligand
TRAIL-R1 = DR4, TRAIL receptor 1
TRAIL-R2 = DR5, TRAIL receptor 2
TRPV1 = transient receptor potential cation channel subfamily V member 1
Trxs = thioredoxins
UPR = unfolded protein response
VCAM-1 = vascular adhesion molecule-1
VEGF = vascular endothelial growth factor
VSMCs = vascular smooth muscle cells
XDH = xanthine dehydrogenase
XIAP = X-linked inhibitor of apoptosis
XO = xanthine oxidase
XOR = xanthine oxidoreductase

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