Redox and oxidant-mediated regulation of apoptosis signaling pathways: immuno-pharmaco-redox conception of oxidative siege versus cell death commitment

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Abstract

The mechanisms controlling apoptosis remain largely obscure. Because apoptosis is an integral part of the developmental program and is frequently the end-result of a temporal course of cellular events, it is referred to as programmed cell death. While there is considerable variation in the signals and requisite cellular metabolic events necessary to induce apoptosis in diverse cell types, the morphological features associated with apoptosis are highly conserved. Free radicals, particularly reactive oxygen species (ROS), have been proposed as common mediators for apoptosis. Many agents that induce apoptosis are either oxidants or stimulators of cellular oxidative metabolism. Conversely, many inhibitors of apoptosis have antioxidant activities or enhance cellular antioxidant defenses. Mammalian cells, therefore, exist in a state of oxidative siege in which survival requires an optimum balance of oxidants and antioxidants. The respiratory tract is subjected to a variety of environmental stresses, including oxidizing agents, particulates and airborne microorganisms that, together, may injure structural and functional lung components and thereby jeopardize the primary lung function of gas exchange. To cope with this challenge, the lung has developed elaborate defense mechanisms that include inflammatory-immune pathways as well as efficient antioxidant defense systems. In the absence of adequate antioxidant defenses, the damage produced is detected by the cell leading to the activation of genes responsible for the regulation of apoptosis, conceivably through stress-responsive transcription factors. Oxidative stress, in addition, may cause a shift in cellular redox state, which thereby modifies the nature of the stimulatory signal and...
which results in cell death as opposed to proliferation. ROS/redox modifications, therefore, may disrupt signal transduction pathways, can be perceived as abnormal and, under some conditions, may trigger apoptosis.

**Keywords:** Antioxidant; Apoptosis; Epithelium; Oxidative stress; Redox; Transcription factors

1. **Introduction**

The regulation of programmed cell death, or apoptosis, can be considered as one mechanism, which is closely associated with cell development and another, which is involved in maintaining cell integrity and homeostasis. The asynchronous nature of cell death is attributed to variable duration and timing of events and the actual propagation of the apoptotic process. The multi-faceted complexity by which apoptosis is controlled requires the coordinated regulation of signaling cofactors, transcription factors and the presence of an extracellular motivation, which is represented by the effect of stimuli such as reactive oxygen and nitrogen species [1–5]. The airway epithelium has versatile roles that are key components of the mechanisms which help maintain and perpetuate the integrity and welfare of this delicate tissue of the lung. The integrity of the airway epithelium is particularly reinforced by a tightly regulated equilibrium existing between cell proliferation/differentiation and degeneration (apoptosis). This review summarizes our recent understanding of the mechanisms that regulate programmed cell death in physiologic and pathologic conditions, while focusing on elaborating pharmaco-redox concepts underlying pathways of redox/oxygen signaling mediating apoptosis.

2. **Biochemical and biophysical properties that characterize the airway epithelium**

Apoptosis, or programmed cell death (also referred to as an ‘orderly cell deletion’), is a genetically controlled mechanism involved in development, maturation and homeostasis [1–5]. The term ‘apoptosis’ is often used interchangeably with ‘programmed cell death’. In the strictest sense, programmed cell death may be applied to other forms of cell death that require gene expression without fulfilling some, or all, of the morphological criteria of apoptosis. Whatever the definition, studies clearly show that apoptosis is genetically regulated. This process is characterized by a variety of cellular changes including loss of membrane phospholipid asymmetry, chromatin condensation, mitochondrial swelling and DNA cleavage. Apoptosis may be induced by stimuli as diverse as hyperthermia, growth factor withdrawal, chemotherapeutic agents, irradiation, cytokines and oxidative stress [2,4,6]. Apoptosis, which has recently emerged as an independent field in biomedical research, is believed to be critically involved not only in the development but also in the regulation of a number of pathophysiological conditions, including cancer, idiopathic pulmonary fibrosis, acquired immunodeficiency syndrome (AIDS), autoimmune-associated diseases, acute respiratory distress syndrome (ARDS) and neurological conditions such as Alzheimer’s, Parkinson’s and multiple sclerosis [7–13]. The integrity of the airway epithelium is regulated by a tight, but dynamic, equilibrium of cell proliferation and degeneration (apoptosis), and plays a crucial role in regulating mucosal defenses, inflammatory responses and the evolution of pathological conditions in the lung [1,14–17].

The alveolar epithelium forms a continuous sheath lining the alveolar air spaces of the lung [5,18]. Concomitant with the development of various lung structures is the cellular differentiation of alveolar type I (ATI) and ATII cells as the epithelium progressively matures. During the first 4 months of gestation, the epithelial lining is more or less columnar to cuboidal [19,20]. By 6 months, ATI and ATII cells can be relatively distinguished in the more localized differentiated zones of pseudo-cuboidal cells. ATI cells are thin, flat and squamous epithelia conspicuous due to their small perinuclear body and long cytoplasmic extrusions; they are developed from the cuboidal cells that line bronchioles and cover most of the alveolar wall in later stages of development. In addition, ATI cells are characterized...
by having a low compliment of organelles, which indicates low metabolic activity, thus reflecting the quiescent nature of these cells [5,19,20]. The morphology of ATI cells provides a large surface area with a small volume, ideal therefore for rapid and efficient gas exchange. ATII cells, on the other hand, are particularly identifiable owing to their granular and cuboidal appearance, a result of the dense packing of cytoplasmic organelles (indicating metabolically active cells) and lamellar bodies (thickly layered organelles that synthesize and store pulmonary surfactants) [19,20]. ATII cells are small in diameter (≈ 400 μm$^3$ in rat and ≈ 900 μm$^3$ in human) but otherwise appropriate for proper gas exchange. Situated in the corners of the alveolar sacs, ATII cells represent little obstruction to gaseous diffusion and are fed by a capillary network. Intracellularly, these cells are richly endowed with cytoplasmic organelles associated with the biosynthesis of surfactant phospholipid and related proteins. The major function of a surfactant, a mixture of proteins and disaturated dipalmitoyl phosphatidylcholine, is to reduce the lung surface tension, thus facilitating lung expansion during inhalation/exhalation. In addition to surfactant secretion, ATII cells regulate xenobiotic metabolism via the activity of cytochrome monoxygenases [5,19,20], serve as thin, permeable entities for gas diffusion and act as a protective barrier against water and electrolyte leakage (trans-epithelial ion movement) [1,5,19,20]. Furthermore, ATII cells participate in the evolution and progression of immunomodulatory functions, such as regulating the expression of adhesion molecules, chemokines and matrix components, and mediating the release and expression of cytokines and other inflammatory mediators [21–28]. Another major function of ATII cells is the continuous upkeep of biochemical and biophysical properties that characterize the alveolar epithelium. The alveolar epithelium is maintained via two unique properties: (i) ATII cells can proliferate to replenish the original stem cell population, thereby giving rise to new ATII cells; and (ii) they can differentiate into ATI cells to facilitate epithelial repair, regeneration and restoration of tissue architecture and function [1,5]. Subsequently, the existence of a dynamic equilibrium between ATII cell proliferation and/or differentiation, which is controlled via a well-defined programmed apoptotic machinery, has tremendous repercussions for regulating the functioning of an integral epithelium within a continuously challenging and active environment [1,5,29,30].

During lung development, the immature mammalian lung undergoes a series of morphological and functional changes that are characterized by cell proliferation, apoptosis and maturation. For example, apoptosis increases during the architectural changes that accompany lung development. The ATII cell population declines for several reasons, including a decrease in ATII proliferation, an increase in ATII degeneration by apoptosis and/or an increase in the terminal differentiation into ATI cells [1,5]. All of these steps potentially contribute to prepare the lung to assume its postnatal role as a gas-exchange organ [5,29]. Lung maturation, for instance, throughout gestation and in the postnatal period, occurs within widely different ranges of oxygen tensions [5,29,30]. In utero, saccularization of the lung and the functional differentiation of the epithelial lining proceed at a pO$_2$ between 23 and 30 Torr (3–5% O$_2$), which is the oxygen transfer capacity of the umbilical vein [29]. During parturition, Na$^+$-driven fluid absorption drains the fluid support within the lung, ventilation begins, and luminal end tidal pO$_2$ rapidly rises and stabilizes in the range of 70–100 Torr (10–12% O$_2$). The successful transition from placental to pulmonary gas exchange therefore causes a four- to fivefold relative hyperoxic shift at the epithelial lining of the distal lung is associated with hormonally regulated perinatal developmental events, such as the expression and production of surfactant, expression of ion-transport pathways and their components, expansion of gas exchange surfaces and alveolarization. Despite a late gestational increase in expressed antioxidant enzymes (AOE), the full complement of antioxidant defenses in the fetal lung remains approximately threefold lower than neonatal lung and sixfold lower than that of adults. It would therefore appear that the epithelial lining of the perinatal lung possesses a depressed capacity for buffering the production of reactive oxygen species (ROS) and, as such, may be acutely responsive to fluctuations in oxygen availability. The foundation is thus laid for an important oxygen-linked signaling event unique to the period immediately following the first breath, thereby modulating the pattern of gene expression in the epithelial lining of the lung [29,30].
The airway epithelium, therefore, is not an inert barrier; it is a major participant in signaling mechanisms during development, differentiation and pathophysiology [29–31]. Any damage to the airway epithelium can adversely affect its normal physiology and regulatory processes. To recapitulate, the major properties of the airway epithelium include the following: (i) a physiological barrier that regulates diffusion and osmosis; (ii) an active integral metabolic function manifested by the synthesis and degradation of chemical components either endogenously produced or exogenously introduced; (iii) an ability to regulate the proliferation, maturation, regeneration and differentiation of the airway epithelium; and (iv) a secretory feature with an inherent capacity to produce mucous, cytokines and chemokines, hormones, growth factors and enzymes. These properties underlie and support the significance of a physiologically competent epithelium; in the case of metabolic failure or noxious damage, a situation may occur that can lead to abnormalities in the normal development and functioning of the airway epithelium and lung [15,20,31,32].

3. Cell death regulation: the immunopharmacologic paradigm of necrosis versus apoptosis

In the last few decades, since the term ‘apoptosis’ was coined [33,34], a vast quantity of work has been performed in search of the cause of the phenomenon it originally alluded to. It became clear, however, that some cells are genetically programmed, or destined, for death during the normal development of multicellular organisms [35]. The general apoptotic model today is one of intercellular signaling molecules operating in intracellular effector systems that balance each individual cell’s progress to either life (survival) or death (apoptosis) (Fig. 1) [5,36]. Apoptosis, first identified as ‘shrinkage necrosis’ [33–36], was originally observed in mature human/vertebrate tissues as a stochastic loss of cells that showed distinctive histopathological morphology and induced a minor inflammatory response. Simply, it was argued that the key tenets of this model state that there is a universal genetic program that governs cell death at different stages of development, that a variety of stimuli can elicit or activate this program, and that, even though

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**Fig. 1.** Diverse potential divergent and convergent pathways regulating the propagation of apoptosis (cell suicide), or programmed cell death.
many divergent transduction mechanisms are involved, apoptosis ultimately requires the activation of a downstream convergent and common pathway [3,4,6,36]. During apoptosis, cells undergo characteristic morphologic changes including condensation and fragmentation of the nucleus, shrinkage of the cytoplasm and formation of apoptotic bodies containing self-enclosed fragments (Fig. 1). These features stand in stark contrast to those that characterize necrosis, the prevailing form of cell death resulting from nonspecific injury such as trauma, exposure to toxins or abrupt deprivation of vital resources necessary for supporting life.

3.1. The distinct forms of cell death: morphological aspects

At least two distinct forms of death are known by which cells, including airway epithelial cells, undergo death: (i) the well-characterized, and usually rapid, necrotic tissue damage induced by physical trauma or other injury, and (ii) a more protracted and morphologically distinct form of cell death that was then termed apoptosis [3,6,33,34,37]. In apoptosis, cells often shrink, dissociate from surrounding cells and undergo cytoplasmic membrane blebbing. The rapid condensation and aggregation of chromosomes and the formation of small apoptotic bodies are major manifestations of cell death. During apoptosis, even as cellular organelles retain their definition for a long time, the nucleus in particular displays a distinctive pattern of heterochromatization and eventual fragmentation. In many, but not all, apoptotic cells, the condensed chromosomes are acted upon by specific nucleases, which cleave the DNA, thereby producing a characteristic ladder of DNA fragments [3,6,37]. Necrotic cell death, on the other hand, is relatively violent and is characterized by cytoplasmic swelling, rupturing of cell membranes, dilatation of the mitochondria and disintegration of subcellular and nuclear components. Conversely, apoptosis is characterized by an ordered series of events that take place over a longer period of time. Although necrosis may be more analogous to random acts of cellular violence leading to cellular murder, apoptosis is more appropriately referred to as cellular suicide. The cell initiates apoptotic death when it senses that its environment or physical state has been vigorously compromised; this is, indeed, the ultimate self-sacrifice (Fig. 1).

3.2. The biochemistry of cell death: molecular aspects

If there is such simple dichotomy in the modes of cell death, perhaps there is more than one basic genetic program for death and more than one final common pathway. Perhaps some ligand-induced cell death could result from confused or inappropriate regulation of gene expression rather than from turning on pre-set genetic programs (Fig. 1). Studies of *Caenorhabditis elegans* development, for example, have contributed significantly to the biomolecular understanding of cell death [3,6,36,37]. Genetic analysis has led to the identification of cellular genes required for programmed cell death during the development of *C. elegans*. The isolation and molecular characterization of *C. elegans death* (*ced*) genes demonstrated that the *ced-3* gene was homologous to the mammalian interleukin-1β-converting enzyme (ICE; Caspase-1) [3,6,36]. ICE was originally isolated from mammalian cells as an enzyme essential for the proper processing and biologic activation of pro-interleukin-1β, a cytokine involved in mediating cellular inflammatory processes [3,22–25,29,30]. The expression of *ced-3* rapidly induces apoptosis, as does ICE, demonstrating that the *ced-3* gene encodes a cysteine protease essential for programmed cell death [3,6,36]. Many of the ICE-like proteases, henceforth referred to as caspases, were subsequently isolated by molecular cloning [36]. The term *caspase* is based on a nomenclature unanimously adopted: *c* reflects a cysteine protease mechanism and *aspase* refers to the ability of these proteases to cleave a protein following an aspartic acid residue [3,6,36]. Many of these caspases contain a conserved sequence, QAC(R/Q)G, required for the catalytic activity of these enzymes [3,6,36]. The activation of caspase proteases has been linked to the aggregation of cell surface receptors when receptor-sensitive target cells are exposed to the appropriate ligand or when the receptors self-aggregate in response to their high cell-surface density [3,6,36]. Therefore, a functional caspase enzyme can be generated following the receptor oligomerization by autocatalysis, or by the action of another alerted caspase. Recent evidence suggested that caspases regulate the process of apoptosis by...
controlling additional cellular processes such as the progression through the well-defined cell cycle and its various regulators [3,6,36].

As noted, multicellular organisms eliminate redundant, damaged, or infected cells by a stereotypic program of cell suicide [3,6,36,39]. The first mammalian regulator of apoptosis emerged when B-cell leukemia/lymphoma-2 (bcl-2), the gene activated by chromosome translocation in human follicular lymphoma [40], was unexpectedly found to inhibit apoptosis and to permit the survival of cytokine-dependent hematopoietic cells, both in a quiescent state and in the absence of exogenous cytokine [41]. Ced-9 of C. elegans and the mammalian Bcl-2 proved to be functional and structural homologues and their survival function is opposed either by close relatives such as Bax, or by distant cousins such as the mammalian Bik, or Nbk and nematode EGL-1 [3,6,36,39]. All members possess at least one of four conserved motifs known as Bcl-2 homology domains (BH-1–BH-4). Most pro-survival members, which can inhibit apoptosis in the face of a wide variety of cytotoxic insults, contain at least BH-1 and BH-2, and those most similar in structure to Bcl-2 have all four BH domains. Pro- and anti-apoptotic family members can heterodimerize and seemingly are capable of titrating one another’s function, suggesting that their relative concentrations may act as a rheostat for the suicide program. Bcl-2 resides on the cytoplasmic face of the mitochondrial outer membrane, endoplasmic reticulum (ER) and nuclear envelope and may register damage to these compartments and affect their behavior, possibly by modifying the flux of small molecules and proteins [42]. On the other hand, Bax, for instance, is found in the cytosol before an apoptotic stimulus, even though, like most other family members, it bears a hydrophobic domain. Biochemical evidence suggests that the pro-survival proteins may function by directly inhibiting the activity of caspases, directly or indirectly preventing the release of cytochrome c from the mitochondria, which, along with ATP, may facilitate structural changes in the procaspase domain, allowing its cleavage and activation [3,6,36]. Bax, Bax-like proteins and other anti-survival proteins may promote apoptosis by cleaving and activating caspases but also can initiate caspase-independent death via channel-forming activity, which could promote the mitochondrial permeability transition or puncture the mitochondrial outer membrane [3,6,36].

4. Redox and oxidant regulation of apoptosis

4.1. Redox dynamic equilibrium as determined by the glutathione/glutathione disulfide couple

‘Reduction-oxidation’ (redox) state is a term often widely adopted in the burgeoning field of free radical research and oxidative stress [29,30,43,44]. The major determinant of redox status in mammalian cells is glutathione (GSH; L-γ-glutamyl-L-cysteinyl-glycine), a tripeptide thiol that couples with its disulfide form (GSSG) as a redox buffer system (2GSH/GSSG; GSSG + 2H+ + 2e− → 2GSH) (Fig. 2) [45,46]. According to Walter H. Nernst’s theory, redox potential can be determined by the Nernst equation, \[ \Delta E = \Delta E^\circ - (RT/nF)\ln Q \], where \( R \) is the gas constant \( (R=8.314 \ J/K \cdot mol) \), \( T \) the temperature [in Kelvin (K)], \( n \) is the number of moles of electrons involved in the reaction, \( F \) the Faraday constant \( (F=9.6485 \times 10^4 \ C/mol) \) and \( Q \) the ratio of GSH/GSSG, thereby giving yield results in volts. Therefore, the Nernst equation at 25 °C (298.15 K), taking into consideration 2.303 as the conversion factor for \( \ln \) into \( \log_{10} \), can be written as \( \Delta E = \Delta E^\circ - (59.1 \ mV/n)\log_{10}Q \). Thus, the Nernst equation for the reduction potential of 2GSH/GSSG couple is:

\[ \Delta E = -240 - (59.1/2)\log(([GSH]^2/[GSSG])) \text{ mV} \]

at 25 °C, pH = 7.0

This ubiquitous nonessential sulfhydryl amino acid and antioxidant thiol plays crucial roles in maintaining intracellular redox equilibrium and in regulating cellular defenses augmented by oxidative stress [29,30,44]. Synthesized by the action of the rate-limiting enzyme γ-glutamylcysteine synthetase (γ-GCS), GSH uniquely provides a functional cysteinyl moiety that is responsible for much of the diverse properties of glutathione. GSH is mainly located within the cytosol at concentrations of 1–10 mM [45–47]. Glutathione participation in the physiology of cellular metabolism reflects the importance of this molecule in intracellular functions. First, GSH is involved in the detoxifi-
cation of highly reactive peroxides (ROOH) by the conjugation of electrophiles and metals through the glutathione-peroxidase coupled reaction, thus acting as an antioxidant. For example, endogenously produced radicals such as hydrogen peroxide (H$_2$O$_2$) are effectively reduced by the selenium-dependent GSH peroxidase in the presence of GSH as a substrate. During this reaction, GSH is oxidized to generate GSSG, which is recycled back to GSH by the action of glutathione reductase (GSSG-RD) at the expense of NADPH/H$^+$, thus forming the redox cycle. The reduction of the glutathione pathway is blocked by the action of 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU). The major source of NADPH/H$^+$ comes from the conversion of glucose, a reaction blocked by dehydroepiandrosterone (DHEA).

4.2. Redox/ROS signaling in apoptosis

Molecular oxygen is an essential component of the mechanisms mediating the derivation of energy in all aerobic organisms [50]. Aerobic energy metabolism involves the process of oxidative phosphorylation, where oxygen serves as the final electron acceptor for cytochrome $c$ oxidase that catalyzes the reduction of oxygen to water. During the process of the aforementioned procedure, the probability that partially reduced and highly reactive metabolites of oxygen will be produced is substantial. These metabolites include superoxide anion (O$_2^-$) and H$_2$O$_2$; the latter,
in the presence of transition metal ions such as iron, is promptly converted to hydroxyl radical (·OH) via what is known as the Fenton-like reaction (Fig. 3) [22,23,50,51]. These highly reactive metabolites of oxygen are often referred to as ‘ROS’ and they are recognized as potentially toxic by-products of cellular metabolism; however, ROS may also participate in cellular signaling processes, often affecting redox equilibrium and gene regulation [29,30,50]. Alternatively, low-level oxidative stress is known to activate kinase cascades and transcription factors, such as activator protein-1 (AP-1), hypoxia-inducible factor-1α (HIF-1α), and nuclear factor-κB (NF-κB) [27,29,30,36,52]. Pathways linked to the generation of ROS, whether they are linked to the mitochondria or other sources, are believed to constitute a vital component of cellular oxygen signaling mechanisms which integrate the expression of genes involved in energy production, oxygen transfer, cellular differentiation and free radical scavenging, with prevailing oxygen tension [29,30]. Thus, the intensity of any form of oxygen-linked signal is governed by (i) the direction of the shift in $pO_2$ (i.e., a relative hypoxia or hyperoxia), (ii) the magnitude of the shift in $pO_2$, (iii) the cellular capacity for generating ROS and (iv) the expression and/or presence of antioxidants. It follows that both the form and the magnitude of the response to any oxygen-based signal depend on whether or not an effective means for buffering the production of ROS is in place.

The major pathways for cell signaling, which involve protein phosphorylation and redox dynamic fluctuations, may have a colossal impact on cellular functions ranging from proliferation and differentiation to regulation of cell cycle events, apoptosis and, under extreme conditions, necrosis (Fig. 4) [30,50,53]. Therefore, oxidizing signals are crucial regulators of cell life and death [3,50,51]. Certainly, the apoptotic process is preferentially triggered with moderate oxidative stimuli, whereas necrosis occurs as a result of a severe insult due to overwhelming oxidative stress. This is predictable since apoptosis requires

Fig. 3. Schematic representation of the pathways leading to the generation of reactive oxygen species (ROS) and their selective dismutation. A number of major cellular enzymes that defend against oxidative stress have been conserved through evolution. Superoxide (O$_2^-$) anion is metabolized via the dismutation reaction $2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$, which is catalyzed by superoxide oxidoreductase dismutase (SOD), a cytoplasmic enzyme that is constitutively expressed, and by a mitochondrial enzyme that is induced in response to oxidant stress. The $H_2O_2$ produced by the dismutation of $O_2^-$ is converted by one pathway to $H_2O$ and $O_2$ by catalase (CAT) in peroxisomes and by glutathione peroxidase (GSH-PX) in the cytoplasm, at the expense of reduced glutathione (GSH), leading to the formation of oxidized glutathione disulphide (GSSG) that is recycled back to GSH by glutathione reductase (GSSG-RD). $H_2O_2$ could be further converted by another pathway involving iron into the hydroxyl radical (·OH), an injurious ROS causing cellular damage. This iron-catalyzed reaction, known as the Fenton-like reaction, is impeded by the iron chelator desferrioxamine (DSF), which is also capable of neutralizing the toxicity of ·OH.
the expenditure of ATP to program and execute the process, while necrosis is initiated when oxidative conditions deplete energy resources [6,51].

4.3. Regulation of apoptosis by oxidants: the paradigm of oxidative siege

Oxidative stress is a biochemical condition that is characterized by the presence of relatively high levels of toxic reactive species, principally consisting of ROS, reactive nitrogen species (RNS), reactive nitrogen oxygen species (RNOS) and unbound adventitious metal ions [3,50,51,54]. The production of ROS, in particular, has been associated with programmed cell death in many conditions such as stroke, inflammation, ischemia, lung edema and neuro-degeneration [55–58]. Many of the chemical and physical treatments capable of inducing apoptosis are also known to evoke oxidative stress. Incubating cells, for instance, with exogenous oxidants, free radicals, or adding redox-active compounds, has been shown to initiate and perpetuate the apoptotic process [55–58]. In addition, the ability of nitric oxide (NO) to rapidly react with the heme group of guanylate cyclase has been used as a message in activating downstream apoptotic cellular pathways [59]. Subsequently, it was proposed that NO plays a major regulatory role in apoptosis, but the molecular mechanism is still unclear. However, low physiological concentrations of NO can in fact inhibit apoptosis, but higher concentrations of NO may be toxic by predisposing cells to tumorigenesis through DNA damage, inhibition of DNA repair, alteration in apoptosis or activation of proliferative signaling pathways [60,61]. Furthermore, both ionizing and UV irradiation are capable of inducing apoptosis by inducing the generation of \( \cdot \text{OH} \) and \( \text{H}_2\text{O}_2 \) [56,57]. Recent studies, moreover, have focused on the role of \( \text{H}_2\text{O}_2 \) in regulating apoptosis. This peroxide is an oxidant that triggers caspase activation and subsequent apoptosis. Of note, \( \text{H}_2\text{O}_2 \)-mediated caspase activation is dependent on the release of cytochrome
from the mitochondria, suggesting a key role for this peroxide in mitochondrial permeability and leakage [36,56]. For example, exposure to low doses (10–100 μM) of H2O2 induces apoptosis in a variety of cell types, thereby directly establishing oxidative stress as a mediator of cell death [53,57]. Catalase (CAT), in addition, prevented H2O2-mediated apoptosis, suggesting that this oxidant plays a key role in the mechanism responsible for triggering the process of apoptosis [53]. However, high doses of H2O2 have been shown to be cytotoxic by triggering a disorderly form of cell death, or necrosis [57,58]. In support of the proposal that H2O2-mediated cell death is dependent on the activation of one or more oxidative stress-regulated pathways, it has been observed that growth factors, such as keratinocyte growth factor (KGF), promote alveolar epithelial cell DNA repair after H2O2 exposure independent of alterations in catalase, GSH or Bcl-2 and Bax proto-oncogenes [62]. For the proposed mechanisms, it has been hypothesized that the oxidation of cellular molecules could trigger a general protection alert system and that these sensors in turn detect and assess the damage, subsequently activating the apoptotic machinery (Fig. 4). Interestingly, antioxidants and GSH precursors confer a protective effect against ROS-mediated injury and subsequent apoptosis, implicating a critical role for ROS in initiating the apoptotic death machinery [28–30,32,35,36,63–70].

Oxidative stress, therefore, results from an oxidant/antioxidant balance in favor of oxidants and this situation certainly has negative consequences in lung pathophysiology [10,13,16,27,48,49,67,71]. The respiratory tract is subject to a variety of environmental stresses and airborne pollutants and extracellular antioxidants provide a major defense to the external surfaces of the respiratory tract epithelium [70]. The highly elevated levels of ROS following exposure to hyperoxia, for example, can overwhelm the antioxidant system of the lung, including the airway epithelium, which then becomes a target of ROS-mediated injury and death [72]. In animal models, prolonged exposure to hyperoxia results in injury and death of both endothelial and epithelial cells in the lung [73,74]. Histological and electron microscopic studies have shown that the earliest signs of hyperoxic cell injury can be detected in capillary endothelial cells [72]. Similar morphometric changes are observed in ATII cells of animals exposed to hyperoxia [72]. Several apoptotic cofactors are presumed sensitive to hyperoxia in the lung; for instance, members of the Bcl-2 family, Bax and Bcl-X, are activated by hyperoxia at the transcriptional level in vivo [74]. In another study, fetal ATII (fATII) epithelial cells were used to evaluate the role of signaling factors involved in oxidative stress-induced apoptosis [75,76]. Bcl-2 proto-oncogene showed a maximum abundance during hypoxia and mild reoxygenation, but declined thereafter with hyperoxia. The Bcl-2 counterpart, Bax, displayed an increasing logarithmic profile with ascending ΔpO2, such that the ratio of Bcl-2/Bax decreased as pO2 increased. The expression of p53, a cell cycle regulator, paralleled Bax abundance. In addition, pretreatment of fATII cells with L-buthionine-(S,R)-sulfoximine (BSO), an irreversible inhibitor of γ-GCS (the rate-limiting enzyme in the biosynthesis of GSH), enhanced Bax and p53 expression over Bcl-2. The GSH analogue, γ-glutamylcysteteinyl-ethyl ester (γ-GCE), down-regulated Bax/p53 concentration but restored that of Bcl-2, thereby increasing the Bcl-2/Bax ratio. The antioxidant and GSH precursor N-acetyl-L-cysteine (NAC) favored Bcl-2 at the expense of Bax/p53, whereas pyrrolidine dithiocarbamate (PDTC), a non-thiol antioxidant, induced Bax against Bcl-2, with a mild effect on p53. Sulfasalazine (SSA), a potent and specific inhibitor of NF-κB, induced Bax at the expense of Bax/p53, whereas pyrrolidine dithiocarbamate (PDTC), a non-thiol antioxidant, induced Bax against Bcl-2, with a mild effect on p53. Sulfasalazine (SSA), a potent and specific inhibitor of NF-κB, induced Bax at the expense of Bax/p53, whereas pyrrolidine dithiocarbamate (PDTC), a non-thiol antioxidant, induced Bax against Bcl-2, with a mild effect on p53. In accordance with the aforementioned observations, O’Reilly et al. [78] reported a crucial role for p53 expression in hyperoxia-mediated DNA damage of pulmonary epithelial cells, which responded by accumulating p53, an indication of epithelial cell-specific gene expression. A classic example of this model is
p53-mediated detection of DNA damage [79,80]. For example, p53-mediated apoptosis is hypothesized to occur by the increased transcription of pro-oxidant factors, thereby leading to caspase activation and apoptosis [29,36]. A proposed modified model of the mammalian cell death pathways involving the intracellular cofactors activated by oxidative stress is given in Fig. 5.

Protection of alveolar epithelial cells and other vital pulmonary and endothelial linings against oxidative stress-mediated death and injury was proposed via the delivery of antioxidant enzyme proteins to the lung (Fig. 4) [81]. The airway administration of superoxide dismutase (SOD) and catalase, for example, protects alveolocytes against hyperoxic-induced injury [81]. Furthermore, overexpression of manganese SOD (MnSOD) protects lung epithelial cells against oxidant injury and co-expression of CAT and MnSOD offers additional protection from hyperoxic injury [82]. However, the importance of dissecting the route from oxidative stress/redox signaling to apoptosis is not restricted to models where cells are exposed to exogenous oxidants, as is the case with the airway epithelium. This might explain, in part, why antioxidant therapy in some occasions is hardly effective against oxidative stress-mediated cell death [81]. Intracellular oxidant production has been detected in cells incubated with a wide range of seemingly independent apoptotic agents and some of these changes are suggested, however, to occur sufficiently early to be intricately involved in the activation of apoptosis [3,36]. The paradox is that, in some instan-

![Fig. 5. Schematic overview of apoptotic signaling cofactors in mammalian cells. Activation of different damage pathways or stimulation of pro-apoptotic signal transduction cascades can have at least two consequences: (i) perturbation of mitochondrial membrane integrity through activation of the permeability transition pore complex (PTPC or megachannel) and/or Bcl-2/Bax complex in the mitochondrial outer-inner membrane contact site, or (ii) primary activation of caspase cascades that act as effectors of the ultimate process of apoptosis.](image-url)
ces, hyperoxia and ROS may inhibit rather than promote apoptosis. For instance, GSH depletion is insufficient to cause maximal mitochondrial ROS production and that there is an early requirement for protease activation and gene expression [55]. This stands in contrast to our observations in prenatal fATII cells, which show maximal elevation in ROS production following GSH depletion, but that the latter process is not well correlated with the onset and degree of cell death [67,75,76]. Interestingly, Franek et al. [83] have recently reported that hyperoxia inhibits oxidant-induced apoptosis and that this inhibition is mediated by NF-κB. In human lung adenocarcinoma A549 cells, pre-exposure to 95% oxygen reduced apoptosis due to sublethal exposure to H₂O₂ and that the onset of inhibition is correlated with the degradation of inhibitory-κB (IκB), the major cytosolic inhibitor of NF-κB, thereby freeing the complex to allow its translocation and activation [83]. The mechanism by which NF-κB may inhibit oxidant-induced apoptosis is still controversial. One possibility is that activation of this transcription factor may alter intracellular redox equilibrium in favor of a reduction environment [30,83]. This notion is unequivocally supported by studies reported by Haddad and colleagues, who showed that an increase in NF-κB activation could be achieved by treating cells with NAC, suggesting a correlation between NF-κB regulation and increased antioxidant capacity. In contrast, inhibition of NF-κB by PDTC favors an oxidative environment by reversing the GSH/GSSG equilibrium ratio [29,30]. This demonstrates that antioxidant treatment effectively uncouples transcription factor activity from the normal pattern induced by changes in oxygen availability in fetal epithelial cells derived from the distal lung. The capacity of the developing lung to mount an adaptive genetic response to hypoxic or hyperoxic environments is, therefore, determined by the interplay between oxygen availability, redox state, cellular compartments (in this case, nuclear and cytosolic) and the glutathione buffering capacity of the alveolar epithelium [29,30,75,76,84].

4.4. Regulation of apoptosis by reductants: the paradigm of redox disequilibrium

It is becoming evident that the redox status of the cell can have complex and multi-layered effects on apoptosis [44,46,65,75,85]. For example, glutathione depletion is associated with the induction of apoptosis through an ordered pathway involving oxidant accumulation in cultured lung fibroblasts [86]. Furthermore, redox status in macrophages is one of the key factors mediating the apoptotic pathway in which glutathione plays a critical role in mediating cell death via NO and ROS [87]. Similarly, apoptosis is closely associated with the modulation of intracellular glutathione levels upon the introduction of polyunsaturated fatty acids into colorectal adenocarcinoma epithelial cells [88]. Interestingly, cells induced to apoptosis extrude glutathione in the reduced form concomitantly with or prior to the development of apoptosis, an event considered even earlier than plasma membrane leakage [89]. Theoretically, then, rescue of cells from apoptosis is achievable by inhibition of GSH extrusion. Ghibelli et al. [89] demonstrated an interesting mechanism of action of specific inhibitors of carrier-mediated GSH extrusion, methionine and cystathionine. These aforementioned inhibitors successfully decreased apoptotic GSH efflux across the intact cell membrane, suggesting the involvement of a specific mechanism in GSH extrusion. In addition, while decreasing GSH efflux, methionine and cystathionine also decreased the extent of apoptosis, although an anti-apoptotic activity could not be demonstrated [89]. These observations indicate that extrusion of GSH precedes and is responsible for the irreversible morpho-functional changes in apoptosis, thus giving a rationale for the development of redox-dependent apoptosis under anaerobic conditions.

A critical first-line antioxidant defense on the airway epithelial surface against ROS and RNS-mediated injury is extracellular glutathione peroxidase (GSH-PX) [29]. Fusion genes of deletion fragments of the GSH-PX gene 5’-flanking region driving a reporter gene conclusively identified a ROS-responsive region, which contained the consensus DNS-binding site for the redox-regulated transcription factor, AP-1 [90]. It was subsequently proposed that increased ROS formation leads to alterations in intracellular and extracellular reducing/oxidizing environments (GSH/GSSG levels). Concomitant with these changes is the induction of GSH-PX as part of up-regulated antioxidant machinery activated in response to oxidative stress. Furthermore, it was postulated that the mitochondrion may act as a principle sensor and that
redox regulation of the release of mitochondrial factors such as cytochrome c is critical to caspase activation and, hence, propagation of apoptosis [43,91,92]. Redox reactions and electron flow through the respiratory chain are the hallmarks of mitochondria. By supporting oxidative phosphorylation and metabolite transport, mitochondrial redox reactions are of central significance for cellular energy conversion [93]. Consistent with this accredited property of the mitochondrion, Voehringer et al. [94] have recently reported a novel gene microarray approach to identify possible redox and mitochondrial elements that control resistance or sensitivity to apoptosis. Within this context, a multigenic program is proposed for determining the sensitivity to apoptosis; this encompasses a canopy of signaling cofactors that mediate the induction of transcripts for genes participating in mitochondrial uncoupling and loss of membrane potential. This program triggers a mitochondrial release of apoptogenic factors and induces caspase cascades. Conversely, cells resistant to apoptosis down-regulate these biochemical pathways, while activating pathways for the establishment and maintenance of high intracellular redox potential by means of elevated glutathione [93,94].

The redox properties of cytochrome c, a ubiquitous heme-containing electron transport protein, confer a crucial clue as to its involvement in regulating apoptosis [95,96]. A novel approach to define the redox properties of cytochrome c was adopted by Sinibaldi et al. [97]. In this study, cytochrome c reconstituted from the recombination of two fragments of horse cytochrome c (the heme-containing N-fragment, residues 1–56, and the C-fragment, residues 57–104) was shown to be redox-sensitive, suggesting a key role for specific residues in regulating the activity of cytochrome c. Following its release from the mitochondrial periplasmic space, cytochrome c forms a protein complex with apoptotic protease activating factor-1 (Apaf-1), caspase-9 and ATP—an entity called apoptosome, which initiates and activates the degradation phase of apoptosis (Fig. 5) [98–100]. Analysis of redox regulation of cytochrome c-induced apoptosis implied that antioxidants, but not oxidant defensive enzymes, block apoptosis and that cytochrome c reductase are able to inhibit the propagation of apoptosis [101]. Subsequently, a major question emerged: ‘Does the redox status of cytochrome c act as a fail-safe mechanism in the regulation of apoptosis?’ It was suggested that cytochrome c only induces programmed cell death if present in the cytoplasm in the oxidized form and that the presence of cytoplasmic GSH maintains cytochrome c in an inactive (reduced) state, thus behaving as a fail-safe mechanism if cytochrome c is released from the mitochondria when apoptosis is not the desired outcome [102]. Standing in a striking contrast to the aforementioned theory, Cai et al. [103] have reported a separation of cytochrome c-dependent caspase activation from redox disequilibrium in cells lacking mitochondrial DNA. Cytochrome c and caspase activation in response to staurosporine treatment is preserved in pα cells. However, unlike the case with wild type cells (pα) in which the oxidation of GSH occurs after the release of cytochrome c, the thiol-disulfide redox status in apoptotic pα cells remained basically unaffected [103]. It is hypothesized that the cellular signaling of caspase activation can be separated from the bioenergetics property of the mitochondria and that the respiratory chain is critical for the initiation of oxidant-mediated apoptosis (Fig. 6).

The effectors of apoptosis, particularly caspases, are redox-sensitive [104–107]. All members of the caspase family include the sequence QACXG, which contains the active site cysteine [107]. This putative active site containing cysteine is sensitive to redox changes. For example, both thioredoxin (TRX) and glutathione have been shown to be required for caspase-3 activity in inducing apoptosis [107,108] and in regulating apoptosis signal-regulating kinase-1 (ASK-1), a mitogen-activated protein kinase (MAPK) that regulates the activation of c-Jun N-terminal kinase (MAPKJNK) and MAPKp38 [109], both of which are involved in mediating apoptosis signaling [110,111]. Cells, therefore, require the maintenance of a strict reducing environment for caspases to function. By this reasoning, apoptosis, at least theoretically, cannot occur in cells subjected to excessive oxidative stress [3,6,36,65]. However, the ability of oxidants to inhibit caspase function needs not be incompatible with oxidant-dependent caspase activation. First, low levels of oxidants appear sufficient for optimal caspase activation, suggesting specific signaling pathways rather than widespread oxidation. Second, the observed ability of cells to repair or replace...
oxidized caspases indicates that the full complement of apoptotic effector molecules can be returned at some time after the initial oxidative stress [104, 107]. Elucidation of the pathways of oxidant-induced apoptosis, therefore, may provide alternate therapies to the scavenging of the initial oxidant in those systems where excessive oxidative stress and redox disequilibrium lead to inevitable cell death (Fig. 7).

The role of GSH in regulating apoptosis has recently emerged to implicate Bcl-2, which is hypothesized to function at a level common to various apoptotic pathways. The mechanism of action of Bcl-2 as an anti-apoptotic entity revolves around the ability of this proto-oncogene to act as an antioxidant [112]. In addition, Veis et al. [113] examined the phenotype of Bcl-2 knockout mice and found that they have pathologies associated with defects in antioxidant pathways. Furthermore, Bcl-2 in overexpressing cultured cells suppresses the formation of ROS when stimulated to undergo apoptosis, an effect concomitantly occurring with the elevation of intracellular GSH [114–116]. Research into the ROS/redox-mediated regulation of Bcl-2 functions has led to a unifying hypothesis that Bcl-2 expression is associated with the enhancement of antioxidant capabilities which suppress or down-regulate oxidative stress signals generated and/or up-regulated during the initiation phase of apoptosis [117]. In support of this theory, it was demonstrated that the antioxidants NAC and PDTC can mimic the protective effect of Bcl-2 and that Bcl-2 expression causes the redistribution of glutathione to the nucleus [118,119]. These observations were reproduced in alveolar epithelial cells where NAC exhibited an ability to modulate paraquat (O2-)-(O2-) induced apoptosis, suggesting that the selective uptake of paraquat by ATII cells and subsequent induction of apoptosis can remove the ability of the epithelium to restore normal tissue architecture and function, thereby leading to lung tissue damage [5,120,121]. We have previously reported that NAC pretreatment of ATII cells induces intracellular GSH accumulation [29,30,67] and favors an increase in Bcl-2 expression at the expense of its counterpart, Bax, in vitro and in the developing lung ex vivo [67,75,76,122]. Although the precise mechanisms involved in the antioxidant potential of Bcl-2 and its regulation of glutathione homeostasis are still unclear, there is strong support for the suggestion that Bcl-2 is directly in-

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**Fig. 6.** Consequences of mitochondrial permeability transition on signaling pathways and apoptosis. Different death-induced cascades employ various signal transduction mechanisms that culminate in the induction of permeability transition. This allows the propagation of signal transduction pathways or mediates redox disequilibrium and bioenergetics catastrophe. The latter properties activate the process of apoptosis through upstream regulation of cytochrome c release and caspase activation.
volved in the regulation of oxidative stress and oxidant-mediated apoptosis.

5. Conclusions and future prospects

Apoptosis research continues apace—a burgeoning field with fascinating and promising outcomes through immunopharmacologic gene therapy and tissue modulation. Apoptosis is a complex and multi-faceted pre-programmed process that involves a plethora of signaling cofactors which span the cell membrane all the way down to the nucleus. Despite this diversity and complexity, apoptotic pathways ultimately converge to cause a process that may well be part of proliferation and differentiation—as in development—for instance, or degeneration—as in pathophysiology, ageing and disease [122–127]. Current strategies are focused on understanding the complex regulation of the apoptotic machinery and on utilizing this knowledge to develop relieving therapies for a variety of human diseases. Despite major progress, however, fundamental, yet unanswered, questions remain to be addressed. From a pharmacologist’s point of view, targeting the myriad of mechanisms that form an integral part of disease development may become one of the most powerful tools in the future of cell death regulation [125–135]. Hopefully, the coming decade will provide improved understanding of the overall process of programmed cell death, as well as the first triumphant successes in translating this understanding into clinical approaches and beneficial therapies [133–137].

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