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Viswanathan Natarajan
Narasimham L. Parinandi *Editors*

Mitochondrial Function in Lung Health and Disease

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Preface

Mitochondria, often referred to as the "powerhouses" of the cell, generate adenosine triphosphate (ATP) by oxidative phosphorylation or OXPHOS, and maintain cellular homeostasis. In addition, as part of their normal function, mitochondria generate reactive oxygen and nitrogen species which are key regulators of mitochondrial function as well as cell cycle, proliferation, apoptosis, and innate immune responses. The mitochondrion consists of the outer membrane, inner membrane, proteins, lipids and mitochondrial DNA that has substantial similarity to bacterial DNA. Mitochondrial morphology is in a dynamic state being modified continuously enabling the organelle to move, fuse, and fission depending on functional requirements of the cell. Mitochondrial diseases are caused by impairment(s) in the mitochondrial electron transport system, and mitochondrial abnormalities have been documented in pathogenesis of neurodegenerative diseases. Compared to other organs such as heart, brain and liver, the lung has fewer mitochondria as the lung relies on glycolysis more than OXPHOS for energy production. The role of mitochondria in normal lung homeostasis and importance of mitochondrial dysfunction/damage in the pathology of lung diseases remain poorly understood. However, there is evidence for mitochondrial biogenesis in inhalational lung injuries along with oxidative stress and mitochondrial dysfunction in sepsis-induced lung injury, COPD and asthma. Further, mitochondrial abnormalities that perturb the reactive oxygen species-, HIF-1-α-, and oxygen-sensitive K^+ channel pathway may contribute to the pathogenesis of pulmonary hypertension and cancer. Therefore, in addition to their well-recognized role in cellular energy production and apoptosis, mitochondria appear to play a role in many respiratory diseases and lung cancer. By focusing on the mitochondrial metabolism, redox signaling and mechanisms of mitochondrial pathways in lung injury, inflammation, repair and remodeling, this volume will facilitate a better understanding of the emerging concepts of targeting mitochondria to alleviate respiratory lung diseases.

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Chapter 1
Role of Mitochondrial Reactive Oxygen and Nitrogen Species in Respiratory Diseases

Harijith Anantha, Prasad Kanteti, Panfeng Fu, Sainath R. Kotha, Narasimham L. Parinandi, and Viswanathan Natarajan

Abstract Mitochondria are key cellular organelles that not only supply cellular ATP but also integrate redox signaling, apoptotic balance, and biosynthetic pathways in the cell. Mitochondrial dysfunction leads to loss of cellular function, and in humans, mitochondrial dysfunction causes numerous pathologies including cancer, cardiovascular disease, neurological disorders, and respiratory diseases. Mitochondria are a major source of cellular reactive oxygen species (ROS), and mitochondrial ROS production is tightly regulated by the various states of electron transport chain and antioxidant systems present within the mitochondria. As accumulation of mitochondria-derived ROS have been linked to several human diseases, a better understanding of mitochondrial ROS signaling and regulation of its production and function is clinically relevant under physiological and pathological situations. Further, as mitochondrial ROS is linked to mitochondrial dysfunction in various human pathologies, targeting mitochondrial ROS with specific antioxidants has been an area of intense

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investigation. Thus, there is considerable evidence for mitochondrial ROS in normal cell function and signaling, and in this review, we discuss recent advances on the generation, regulation, and targeting of mitochondrial ROS.

Keywords Mitochondria • Oxidative stress • ROS • RNS • Respiratory diseases

Introduction

Mitochondria, often referred to as the "powerhouse of the aerobic cell," play a central role in cellular functions in normal physiology and pathophysiology of various human diseases including atherosclerosis, diabetes, tumorigenesis, inflammation, and mitochondrial disorders. While the origin of mitochondria in eukaryotes is controversial, mitochondria share several features with prokaryotes. The endosymbiotic theory proposes the generally accepted notion that mitochondria originated as an independent organism that took up residence in eukaryotic cells as endosymbionts [1]. Mitochondria regulate various metabolic functions and generate >95 % of the required metabolic energy that is driven by both nuclear and mitochondrial genomes. Of the 37 genes encoded by the mitochondrial genome, 24 are dedicated to processing 13 key genes essential to oxidative phosphorylation (OXPHOS) and energy production.

In addition to generating ATP, mitochondria are an important source of cellular reactive oxygen species (ROS) and reactive nitrogen species (RNS). In recent years, both ROS and RNS have been recognized as important second messengers and signaling molecules involved in the regulation of immunity, differentiation, autophagy, and aging [2, 3]. Mitochondrial ROS production is tightly regulated and is generated by one electron reduction of molecular O_2 to yield superoxide ($O_2^{-\cdot}$) that is subsequently converted to hydrogen peroxide (H_2O_2) by mitochondrial manganese-dependent superoxide dismutase (SOD2). $O_2^{-\cdot}$ generated within the inner mitochondrial membrane is not diffusible; however, H_2O_2 produced by SOD2 easily negotiates membranes and enters cytoplasm where it enters into several oxidative reactions. It gets decomposed to H_2O and O_2 by catalase or utilized by glutathione peroxidase or other peroxidases to oxidize their substrates.

In the past decade, a growing body of evidence emerged demonstrating the importance of mitochondrial ROS-dependent signaling in regulating cellular functions mentioned earlier. However, the role of mitochondria in normal lung homeostasis and its importance in the pathology of lung diseases remain poorly understood. There is evidence for mitochondrial biogenesis in inhalational lung injuries along with oxidative stress and mitochondrial dysfunction in sepsis- and asbestos-induced lung injury, COPD, and asthma. Further, mitochondrial abnormalities that perturb the reactive oxygen species-HIF-1-α-O_2-sensitive K^+ channel pathway may contribute to the pathogenesis of pulmonary hypertension and cancer. Therefore, in addition to their well-recognized role in the generation of cellular energy and apoptosis, mitochondria appear to play a role in many respiratory diseases and lung cancer. In this chapter, the role of mitochondrial ROS signaling pathways in lung injury, inflammation, repair, and remodeling will be addressed.

Generation of Mitochondrial Reactive Oxygen and Nitrogen Species

ROS and RNS have been implicated in several human pathologies, and it is important to understand the different sources of ROS and RNS and their relative contribution to underlying disease. The term ROS refers to a variety of reactive molecules derived from O_2 such as $O_2^{-\cdot}$, hydroxyl radical ($OH^{\cdot}$), or H_2O_2. Similarly, RNS refers to reactive species derived from N_2 and O_2 that include peroxynitrite ($ONOO^-$) and nitric oxide ($NO^{\cdot}$). Of the various reactive species, $OH^{\cdot}$ and $ONOO^-$ are the most reactive and cytotoxic, while $NO^{\cdot}$ and H_2O_2 are less reactive. Mammalian systems utilize multiple pathways for the generation of these reactive species. These include mitochondrial electron transport chain, NADPH oxidase (NOX 1–5), cytochrome P450 enzymes, cyclooxygenases, lipoxygenases, amino acid oxidases, and xanthine oxidase [4–9]. Most ROS are generated in mammalian cells by the mitochondrial respiratory chain [10–12] and NOX proteins [6–8, 13]. Additionally, mitochondrial respiratory chain can produce $NO^{\cdot}$ [14], which on interaction with $O_2^{-\cdot}$ forms $ONOO^-$, a powerful oxidant. Both ROS and RSN can oxidize macromolecules such as proteins, nucleic acids, and lipids, and RSN also can modulate nitration or nitrosation of cellular thiols, proteins, and polyunsaturated fatty acids [15–17].

$O_2^{-\cdot}$ generation within the mammalian mitochondrial matrix is governed primarily by O_2 concentration and levels of reduced electron donors. At least three modes of $O_2^{-\cdot}$ production by isolated mitochondria have been described that then leads to extensive H_2O_2 efflux. These processes depend on (1) NADH/NAD^+ ratio in the matrix [18], (2) coenzyme Q pool in conjunction with a maximal proton-motive force with no net ATP synthesis [19], and (3) mitochondria close to state 3 and making ATP [12]. Generation of $O_2^{-\cdot}$ has been extensively investigated using isolated mitochondria from liver or heart, and at least eight mitochondrial respiratory chains in the different sub-compartments of mitochondria have been identified [20]. Of the eight respiratory chains, at least four multimeric integral membrane protein complexes (complexes I–IV), coenzyme Q (CoQ), and cytochrome c have been identified and well characterized. Complex I (NADH-ubiquinone oxidoreductase) catalyzes the transfer of electrons from NADH to CoQ, which is accompanied by translocation of protons from the matrix to intermembrane space. Similarly, complex II (succinate dehydrogenase) accepts electrons from succinate. There is now evidence that both complexes I and II are capable of generating $O_2^{-\cdot}$. Electrons from complex II move down an electrochemical gradient through CoQ to complex III (ubiquinol-cytochrome c oxidoreductase), which is responsible for $O_2^{-\cdot}$ generation in the intermembrane space along with glycerol 3-phosphate dehydrogenase. Finally, electrons move from cytochrome c to complex IV (cytochrome c oxidase), which uses four electrons to reduce molecular oxygen to water (Fig. 1.1). $O_2^{-\cdot}$ generated at complex I and III can be converted to H_2O_2 by MnSOD or CuZnSOD that resides in mitochondrial matrix or intermembrane space/cytosol, respectively. H_2O_2, unlike $O_2^{-\cdot}$ is freely diffusible and moves across mitochondrial membranes into the cytosol. H_2O_2 is scavenged by glutathione peroxidase in the mitochondrial matrix or

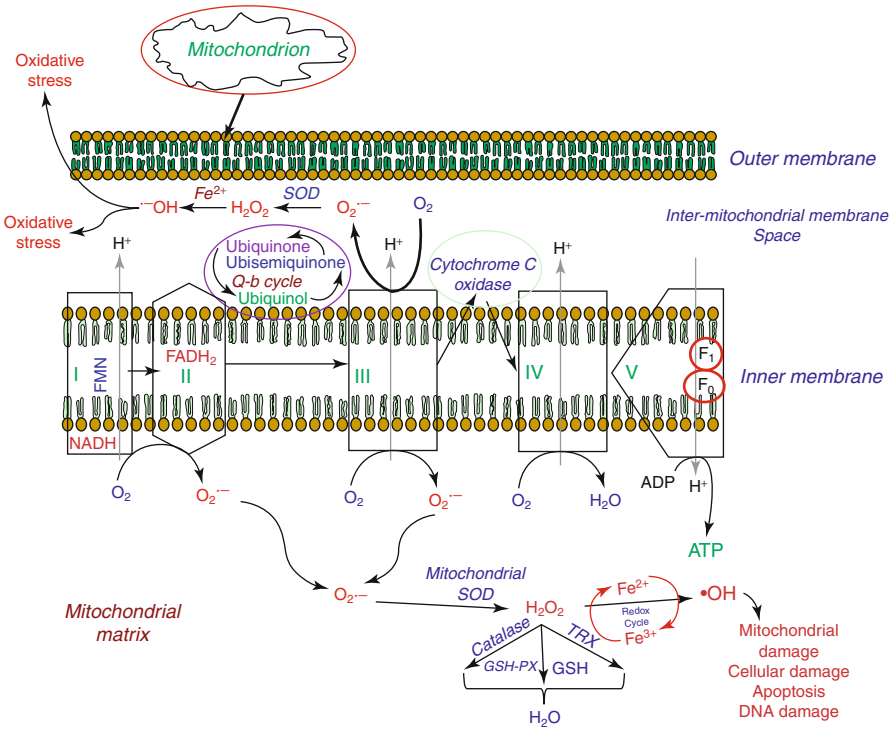

Fig. 1.1 Pathway and sites of mitochondrial oxidative phosphorylation and ROS formation. Molecular oxygen is activated at complexes I, II, and III to superoxide anion ($O2^{\cdot-}$) in the mitochondrial inner membrane. Superoxide anion ($O_2^{\cdot-}$) is dismutated into hydrogen peroxide (H_2O_2) by the mitochondrial superoxide dismutase (*SOD*). Iron (Fe) that is redox-active participates in the formation of highly reactive hydroxyl radical ($^{\cdot}OH$) from H_2O_2. Thus, the ROS ($O_2^{\cdot-}$, H_2O_2, and $^{\cdot}OH$) generated by mitochondria contributes to oxidative stress which leads to mitochondrial dysfunction, cellular damage, DNA damage, and apoptosis. Antioxidant enzymes such as catalase, glutathione peroxidase (*GSH-PX*), and thioredoxin (*TRX*) detoxify H_2O_2 that is aided by glutathione (*GSH*) as a cofactor. *NADH* nicotinamide adenine dinucleotide, *$FADH_2$* flavin adenine dinucleotide, reduced, *Q-b cycle* ubisemiquinone-cytochrome b cycle

by catalase in the cytosol. Thus, $O_2^{\cdot-}$ generated at different sites of mitochondrial respiratory chain may have different physiological and pathophysiological functions, which require further studies.

In addition to $O_2^{\cdot-}$ and H_2O_2, mitochondria is also capable of producing RNS such as $NO^{\cdot}$, generated via breakdown of arginine to citrulline catalyzed by a family of NADH-dependent nitric oxide synthases (NOS). There are at least four isoforms of NOS in mammalian cells: the endothelial constitutive isoform (eNOS) or NOS3, an inducible isoform (iNOS) or NOS2 that is expressed in several cells in response to proinflammatory stimuli and a neuronal isoform (nNOS) or NOS1, and the mitochondrial isoform, mtNOS [21–23]. The existence of mtNOS is still controversial, and recently, another mitochondrial pathway that uses respiratory cytochrome c oxidase as a NO_2 reductase to generate $NO^{\cdot}$ has been shown in rats, plants, algae, yeast,

mouse brain mitochondria, and human endothelial cells [11]. This pathway, unlike NOS pathway, is oxygen independent and activated by hypoxia or anoxia. While $O_2^{-\cdot}$ is the dominant ROS generated under normoxia, $NO^{\cdot}$ is the major reactive free radical species produced under anoxic conditions, which can combine with $O_2^{-\cdot}$ to form $ONOO^-$. Thus, mitochondria-generated ROS and RNS can regulate mitochondrial activity and function by modulation of mitochondrial fusion, fission, mtDNA damage, lipid peroxidation, and electrical gradient [24–26].

Detoxification of Mitochondrial ROS

ROS production in mammalian cells is highly regulated due to its cytotoxicity. The rates of mitochondrial ROS production and accumulation are balanced through mechanisms that regulate electron transfer in respiratory chain and scavenging of excess ROS. Mammalian cells have evolved a number of antioxidant defense mechanisms such as SOD, catalase, glutathione peroxidase, peroxiredoxins, and thioredoxins to detoxify or scavenge excess mitochondrial and non-mitochondrial ROS.

1. *Superoxide dismutase (SOD)*: SOD catalyzes the dismutation of $O_2^{-\cdot}$ to H_2O_2 (Fig. 1.2). There are three isoforms of SOD in mammalian cells including SOD1 (CuZnSOD), SOD2 (MnSOD), and SOD3 (extracellular SOD). Of the three isoforms, SOD2 is exclusively expressed in the mitochondrial matrix [27]. In contrast to SOD1 and SOD2, genetic knockdown of SOD2 causes early neonatal death in mice [28] and endothelial deficiency and dysfunction in apolipoprotein E (ApoE)-deficient mice [29].
2. *Catalase*: The enzyme catalase, which is primarily localized in peroxisomes, catalyzes the dismutation of H_2O_2 to water and O_2 (Fig. 1.2). At least three types of catalases have been described, which include the classic Fe heme enzymes, manganese (Mn) enzymes, and the catalase-peroxidases [30]. Although catalase is a key enzyme involved in dismutation of mitochondrial and non-mitochondrial H_2O_2, knockdown of catalase in mice has no detrimental developmental effects; however, they showed differential sensitivity to oxidant injury. It therefore appears that catalase is dispensable and other detoxifying pathways can compensate [31].
3. *Glutathione peroxidase:* Glutathione peroxidase (GPx) reduces lipid hydroperoxides to their corresponding alcohols and further to H_2O_2 to water (Fig. 1.2). GPx1-4 is a family of isoenzymes homologous to the selenocysteine GPx1 and use reduced glutathione (GSH) as a co-substrate in the reduction of lipid hydroperoxides or H_2O_2. Not all GPxs use GSH nor do they contain selenocysteine at the active site; however, these are thioredoxin-dependent peroxidases containing a redox-sensitive cysteine or a selenocysteine. GPx1 is the most abundant GPx isoform present in all cells and primarily localized in the cytosol, mitochondria, and in peroxisomes of some cells [32, 33]. In addition to reducing H_2O_2 and lipid hydroperoxides, GPx1 may also act as a peroxynitrite reductase [34]. However, the in vivo role of GPx1 in modulating peroxynitrite levels is unclear, but lack of

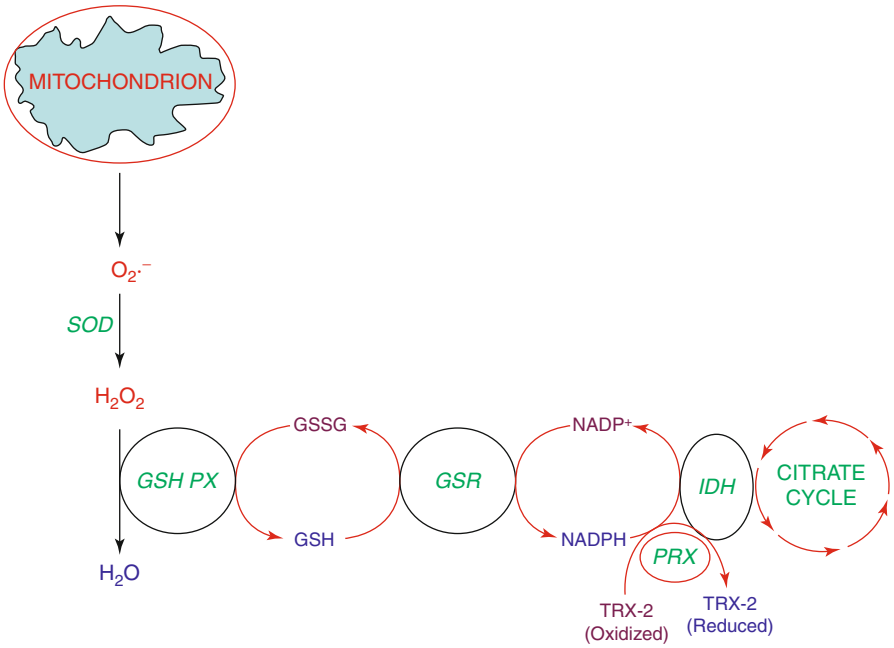

Fig. 1.2 ROS detoxification by antioxidant enzymes and GSH cycling in mitochondria. Superoxide anion (O_2^{-}) generated by mitochondria is dismutated by mitochondrial superoxide dismutase (*SOD*) into hydrogen peroxide (H_2O_2) which in turn is converted into water (H_2O) by the mitochondrial glutathione peroxidase (*GSH-PX*) where in glutathione (*GSH*) acts a cofactor. GSSG, the oxidized form of GSH, is regenerated back into GSH by the action of GSSG-reductase (*GSR*) with the participation of NADPH that is generated by isocitrate dehydrogenase (*IDH*) in the citric acid cycle (tricarboxylic acid cycle). Peroxiredoxin (*PRX*) regenerates oxidized thioredoxin-2 (*TRX-2*) to reduced TRX-2 that is also aided by NADPH

GPx1 enhances survival to peroxynitrite [35] through an as yet undefined mechanism. Knockdown of GPx4, but not GPx1, is embryonically lethal [36]; however, GPx1 knockout mice exhibit susceptibility to ischemia-reperfusion injury in mouse myocardium [37]. The effect of knockdown of GPX1 in mice on mitochondrial ROS production is, however, unclear.

4. *Thioredoxin:* Thioredoxins (Trxs), with a dithiol/disulfide active site (CGPC), are the major cellular protein disulfide reductases [38], and serve as electron donors for enzymes such as ribonucleotide reductases, thioredoxin peroxidases (peroxiredoxins), and methionine sulfoxide reductases [39]. Trx isoforms are present in most organisms; trx1 localizes within the cytosol and translocates to the nucleus during oxidative stress, whereas trx2 is exclusively located in the mitochondria [40]. Trxs are critical for redox regulation of protein function and signaling via thiol redox control. A growing number of transcription factors including NF-kB or the Ref-1-dependent AP1 require Trx reduction for DNA binding. The cytosolic mammalian Trx, lack of which is embryonically lethal, has numerous functions such as defense against oxidative stress, control of growth, and apoptosis.

It is also secreted and has co-cytokine and chemokine activities. Trx reductases of higher eukaryotes are larger (112–130 kDa), selenium-dependent dimeric flavoproteins with a broad substrate specificity that also reduce nondisulfide substrates such as hydroperoxides, vitamin C, or selenite. Mammalian mitochondrial Trx is a monomer of approximately 12 kDa, while mammalian mitochondrial Trx reductase is a selenoprotein homodimer of a 55 kDa subunit. A function of the mitochondrial Trx system is as an enzyme that detoxifies the hydrogen peroxide generated by the mitochondrial metabolism. It is the electron donor for mitochondrial peroxiredoxin. Trx2 deficiency is embryonically lethal at E10.5 d, which coincides with the maturation of mitochondrial function.

5. *Peroxiredoxin:* Peroxiredoxins (Prxs) are a family of six small non-seleno thiol-specific peroxidases and are important regulators of cellular ROS. Prx III is localized in the mitochondria, while Prx V is located both in the mitochondria and peroxisomes [41]. All Prxs reduce H_2O_2 and organic hydroperoxides to H_2O and alcohol, respectively, using reducing equivalents from thiol-containing proteins such as Trxs [42]. All the three systems, GPX/GR, Trx/TrxR, and Prx, rely on NADPH as a source of reducing equivalents and mitochondrial NADPH can be regenerated by NADH-supported reduction of NADP+ via energy-dependent trans-hydrogenation. In the mitochondria, the NADPH reduction is carried out by three mitochondrial enzymes: isocitrate dehydrogenase, malic enzyme, and transhydrogenase [43]. As mitochondrial pools of NADPH and GSH are rather large [44, 45], prolonged ROS generation will depend on the antioxidant defense systems that require regeneration of NADPH and GSH. In mice, overexpression of mitochondrial Prx III protected against left ventricular remodeling and myocardial infarction [46]. Thus, mitochondrial redox homeostasis is achieved through cooperation between GSH/GSSG redox coupling in conjunction with redox proteins, GPx, Trx2, and Prx III.
6. *Nuclear factor erythroid 2-related factor 2:* Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox-sensitive transcription factor that is activated in response to oxidative stress and upregulates antioxidant and phase II detoxification enzymes. Nrf2 is activated by oxidizing extracellular conditions that leads to enhanced mitochondrial ROS production, dissociation of Nrf2 from Keap1, and translocation of Nrf2 to the nucleus, binding to the ARE promoter sequences of cytoprotective genes resulting in induction and expression of antioxidant and anti-inflammatory proteins. A potential link between Nrf2, Trx2, and mitochondrial ROS has been recently described [47]. Nrf2 activation can be inhibited by Trx2 overexpression resulting in decreased mitochondrial ROS [48]. Further support comes from decrease in the expression of genes regulated by Nrf2 [49]. Thus, it appears that extracellular redox states mediate Nrf2 signal transduction through ROS generated in the mitochondria and may be regulated by relative concentrations of Trx2. It is noteworthy that other signal transduction pathways, such as TNFα signaling, are also reliant on mitochondrial ROS production, and these studies have shown Nrf2/Trx2 to function as a modulator of signal transduction [50]. Since ROS production is increased as a consequence of oxidizing extracellular conditions and the inhibition of ROS generation decreases Nrf2

activation, it is therefore possible that ROS may be directly responsible for modification of Keap1 enroute to the activation of Nrf2. However, it is currently unknown whether mitochondrial ROS act directly on the Keap1/Nrf2 system or whether mitochondrial ROS affect other regulatory machinery that may indirectly affect signal transduction through (de)activation of other redox-sensitive components. Since oxidant stress conditions are evident in the pathogeneses of many respiratory diseases, furthering our understanding on the mechanistic control of extracellular-induced Nrf2 activation may lead to the development of potential therapeutic interventions. A detailed description of regulation of mitochondrial function by Nrf2 is provided in the accompanying chapter.

7. *Sirtuins:* Sirtuins, NAD+-dependent histone/protein deacetylases, catalyze the hydrolysis of acetyl groups from the side chain amino group of lysine residues in proteins. The deacetylation requires NAD+ and generates nicotinamide and 2′-*O*-acetyl-ADP-ribose [51]. Seven sirtuins members have been identified in mammals, SIRT1–7; SIRT3, 4, and 5 are primarily located in the mitochondria [52]. SIRT1, 6, and 7 are found in the nucleus, whereas SIRT2 is cytosolic, with robust deacetylase activity. There is strong evidence that supports a role for SIRT1 in mounting a response to oxidative stress by directly deacetylating several transcription factors that regulate expression of antioxidant genes. Notably, SIRT1 activates several members of the FOXO family of transcription factors which promote the expression of stress response genes including SOD2 [53–55]. SIRT1 also promotes mitochondrial biogenesis by activating peroxisome proliferator-activated receptor co-activator 1-α (PGC-1α) [56], which increases mitochondrial mass and upregulates the expression of oxidative stress genes including GPx1, catalase, and MnSOD [57]. Also, SIRT1 inactivates the p65 subunit of NF-κB through direct deacetylation. NF-κB inhibition suppresses the inducible iNOS and nitrous oxide production and thus may lower the cellular ROS load [58]. Mitochondrial SIRT3 deacetylates and activates several enzymes that are critical in maintaining mitochondrial and cellular ROS levels. SIRT3 deacetylates SOD2 at two important lysine residues to boost its catalytic activity, and the deletion of S1RT3 results in loss of catalytic activity of SOD2 [59]. There is evidence that SIRT3 plays an important ameliorative role in preventing the pathological response to pressure overload and aging-associated decline in cardiac function [60]; however, the role of SIRT3 in mitochondrial ROS-dependent respiratory diseases is yet to be defined.
8. *Mitochondria-targeted antioxidant therapies:* Given the central role of mitochondria and mitochondrial ROS in human pathologies, several natural antioxidants such as vitamin C, vitamin E, rottlerin, curcumin, ginsenoside Rb1, and epoetin delta have been investigated both in vitro and in vivo for their antioxidant efficacy, and most of these were not found to be effective in attenuating mitochondrial ROS production in response to an environmental stimulus [61]. In the last two decades, there has been considerable advancement in the development of mitochondria-targeted small molecule antioxidants that exhibited reduced mitochondrial oxidative damage and partially prevented decline in mitochondrial function in some of the pathologies. Several of the small molecule

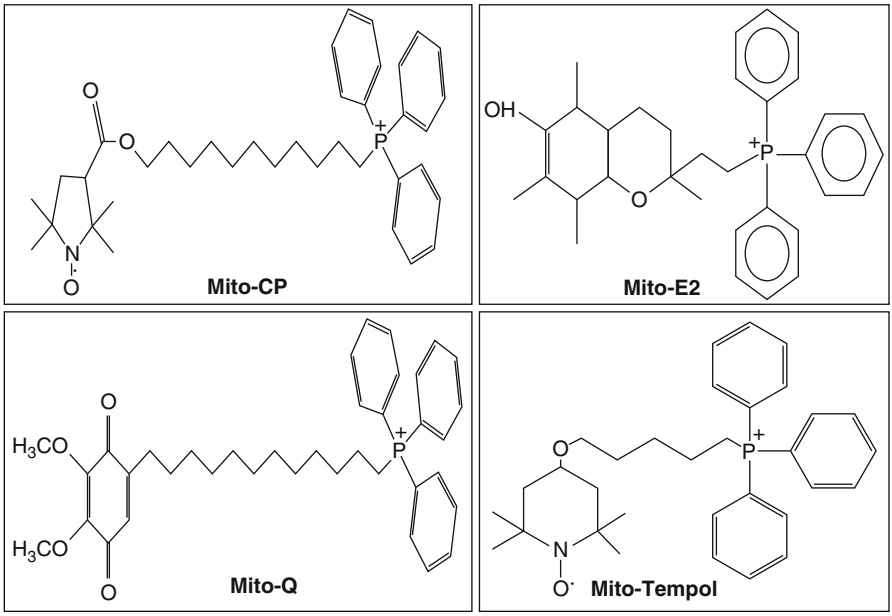

Fig. 1.3 Mitochondrial-targeted antioxidants. *Mito-CP* mito-carboxy proxyl, *Mito-E2* conjugated α-tocopherol moiety to the lipophilic triphenylphosphonium cation (TPP+), *Mito-Q* conjugated ubiquinol moiety of coenzyme Q to the lipophilic triphenylphosphonium cation (TPP+), *Mito-Tempol* 2,2,6,6-tetramethyl-4-[5-(triphenylphosphonio) pentoxy]piper-idin-1-oxy bromide

antioxidants such as alpha-tocopherol, ubiquinone, piperidine nitroxide, and CP have been conjugated to the lipophilic cation, triphenylphosphonium (TPP), to generate mitochondria-targeted small molecule antioxidants Mito-E2, Mito-Q, Mito-CP, and Mito-TEMPOL, respectively (Fig. 1.3).

(a) *Mito-Q:* Among the four mitochondria-targeted small molecule antioxidants, several studies have focused on protective effects of Mito-Q in animal models of human diseases [62, 63]. Mito-Q administered to rats in their drinking water protected against ischemia-reperfusion in the heart, tissue damage, and mitochondria dysfunction [64, 65]. Moreover, Mito-Q administration prevented organ damage [66] and reduced oxidative stress, IL-6 release, and the levels of biochemical markers of endotoxin-induced cardiac dysfunction [67, 68].

(b) *Mito-CP:* In addition to Mito-Q, another TPP-conjugated antioxidant, mitochondria-targeted carboxy proxyl (Mito-CP), has shown promising therapeutic potential against cisplatin-induced nephropathy [69], suppression of medullary thyroid carcinoma cell survival in vitro and in vivo [70], and inhibition of PRx3 and FOXM1 in malignant mesothelioma cell viability [71]. Mito-CP prevented cisplatin-induced mitochondrial injury and dysfunction, renal inflammation, tubular injury, and apoptosis by attenuation of oxidative and nitrative stress mediated by cisplatin [69]. However, Mito-CP

stimulated mitochondrial ROS production in medullary thyroid carcinoma cells [70], and malignant mesothelioma cells [71]. These studies suggest that the effect of Mito-CP to scavenge or generate mitochondrial ROS depends on the cell type and dose of Mito-CP used as vitamin C mediates prooxidant effects via Fenton reaction in cells depending upon the balance between concentration and availability of metal ions [72].

(c) *SkQ1:* The mitochondria-targeted antioxidant, plastoquinonyl-decyltriphenylphosphonium (SkQ1), is a conjugate of a lipophilic decyltriphenylphosphonium cation with plastoquinone, quinine, originally discovered in the electron transfer chain of chloroplast [73]. SkQ1, at nanomolar concentrations, exhibits significant antioxidant efficacy both in vitro and in vivo in animal models of ischemia-reperfusion injury in kidney and development of retinopathy [74, 75]. The therapeutic action of SkQ1 on retinopathy in OXYS rats is linked to normalization of gene expression of vascular endothelial growth factor (VEGF) A and pigment epithelium-derived factor (PEDF). The molecular mechanisms of SkQ1-mediated effects are yet to be defined; however, it is clear that SkQ1 is more potent that Mito-Q in increasing survival and health span in animal models of defined pathologies mediated by oxidative stress. The efficacy of SkQ1 that targets on respiratory diseases is largely unknown.

(d) *Mitochondria-targeted cell permeable small peptide antioxidants:* A number of small, cell permeable, antioxidant peptides, also known as SS (Szeto-Schiller) peptides, that have a sequence motif that targets them to inner mitochondrial membrane have been reported [76]. These SS peptides (SS-01, SS-02, SS-31, and SS-20) of <10 amino acid residues possess a unique aromatic-cationic sequence motif, which alternates between aromatic and basic residues, enabling them to freely permeate cells in an energy-independent manner [77]. The SS peptides have been studied in vivo and in vitro for protection against oxidative cell death and aging. In neuronal cells, SS-31 offered protection against H_2O_2-induced cell death [78]. Similarly, both SS-02 and SS-31 protected cultured neuronal and other cell types against environmental toxicants such as 3-nitropropionic acid-, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-, tert-butylhydroperoxide-, and hypochlorous acid-induced mitochondrial dysfunction, ROS generation, and cell death [79, 80]. These cell permeable SS peptides also exhibited beneficial effects in animal models of diseases linked to elevated ROS production and appeared to have least toxic side effects [81, 82]. Additionally, the XJB and related peptides that belong to hemigramicidin-TEMPO compounds were shown to inhibit actinomycin D-induced ROS generation, cardiolipin peroxidation, and apoptosis in mouse embryonic cells [83, 84] and protect cells in culture against radiation damage [85, 86]. The mechanism of action of cell permeable peptides is presumably via antioxidant action, and further studies are required to evaluate the efficacies of these peptides against respiratory diseases.

Mitochondrial ROS in Pulmonary Disorders

Mitochondrion is a key organelle that draws out the latent energy present in substrates such as glucose, fatty acids, and amino acids to generate utilizable energy in the form of ATP. The bioenergetic status in pulmonary disorders is associated with increased ROS generation, antioxidant depletion, and mitochondrial dysfunction leading to organ dysfunction. A better understanding of the association between mitochondrial ROS production, mitochondrial dysfunction, and the severity and outcome of the pulmonary disorder in question is essential for therapeutic strategies targeting mitochondria.

1. *Acute lung injury:* Acute lung injury (due to sepsis or ventilator-induced lung injury) and subacute lung injury (due to ionizing radiation-induced lung injury) share profound increases in vascular permeability as a key element driving increased morbidity and mortality. There is evidence demonstrating a potential link between sepsis and mitochondrial dysfunction both in septic patients and animal models of sepsis. Ultrastructural abnormalities in mitochondrial morphology including irregular cristae were reported from liver and skeletal muscle biopsies obtained from septic patients who died in ICU [87]. Similarly, in animal models of sepsis, significant alterations of mitochondrial ultrastructure and abnormalities have been described [88–91]. One postulated mechanism is changes in mitochondrial function due to inhibition of mitochondrial respiratory chain, which may contribute to a decrease in oxygen utilization in acute lung injury [92]. Recent studies have identified the involvement of mediators such as TNF-α, ROS, NO, and peroxynitrite in the inhibition of mitochondrial dysfunction [93]. TNF-α, induced in macrophages and lymphocytes by endotoxin, plays a major role in sepsis-mediated lung inflammation and injury via increase in mitochondrial Ca^{2+} and ROS production and signaling [94, 95]. In severe sepsis, there is evidence for a massive influx of extracellular Ca^{2+} via Ca^{2+} release-activated (CRAC) channel or by opening of voltage-, receptor-, or IP_3-operated channels, which is partly the case in mitochondria leading to swelling, mitochondrial dysfunction, and ultimately cell death. While therapies for sepsis are limited, a substantial body of evidence from animal models suggests a beneficial role for mitochondrial targeting with antioxidants such as Mito-Q against endotoxin-mediated sepsis [66]. Further, mitochondria-targeted antioxidants reduced IL-6 release and oxidative stress and improved mitochondrial function in a rat model of acute sepsis [67]. These studies suggest that antioxidants targeted to mitochondria may be an important therapeutic approach in the management of sepsis and multiple organ dysfunction syndrome. A recent study on soluble TNF-α-mediated shedding of the TNF-α receptor 1 ectodomain via increased mitochondrial Ca^{2+} and ROS and TNF-α-converting enzyme may limit inflammatory response in sepsis [94]. The importance of mitochondrial dysfunction in sepsis was also demonstrated in studies related to administration of bone-marrow-derived stromal cells (BMSCs). Exogenous administration of BMSCs

offered protection in mouse models of sepsis-induced acute lung injury [96, 97]. The protection was attributed to Cx43-dependent alveolar attachment and transfer of mitochondria from BMSCs to alveolar epithelial cells [98]. Future studies on mechanisms regulating mitochondrial function may lead to targeted therapeutic approaches for treating sepsis-induced pulmonary inflammation and injury.

2. *Allergic bronchial asthma:* Asthma is a complex lung disease characterized by airflow obstruction, airway hyperresponsiveness, and airway inflammation. Exposure to allergens and environmental pollutants induces innate and acquired immune systems to release proinflammatory and bioactive mediators which lead to recruitment and activation of various inflammatory cells such as eosinophils, mast cells, macrophages, neutrophils, lymphocytes, and platelets [99]. Recent studies have revealed the involvement of mitochondria and mitochondrial dysfunction in asthma pathogenesis. Mitochondrial DNA changes and mutations play a role in the development of asthma. Polymorphisms or haplotype differences in the mitochondrial genome may influence the severity of asthma in humans [100, 101]. Human mitochondrial DNA defects have been observed to accumulate with age and in age-related neurodegenerative diseases such as Alzheimer's and Parkinson's, which are induced by free radicals and ROS. Although no mitochondrial DNA changes are known to date, age-dependent mitochondrial defects may play a critical role in determining the susceptibility to asthma in the elderly. In addition to mitochondrial DNA, mitochondrial tRNA mutations along with specific rRNA mutations have been significantly more frequent in asthmatic patients compared to control subjects suggesting a role for mitochondrial genetic background in asthma pathogenesis [102]. Involvement of mitochondrial ROS in the pathogenesis of bronchial asthma has received considerable attention in recent years. Several lines of evidence in animal models of asthma and human asthma suggest that ROS released by activated macrophages and granulocytes induces oxidative damage in mitochondria of airway epithelial cells, thereby increasing mucus secretion and epithelial permeability [103–105]. Inhalation of allergens and environmental pollutants induces airway inflammation, releasing proinflammatory mediators such as histamine, PGE2, leukotrienes, and NO. These mediators enhance airway smooth muscle contraction as well as recruit and activate various inflammatory cells into the alveolar space [99], which release ROS, peroxynitrite, and lipid peroxidation products, and they further damage the epithelium. Moreover, the asthmatic epithelium is more susceptible to apoptosis mediated by ROS, probably due to diminished antioxidant levels and decreased endogenous antioxidant enzymes in the peripheral tissues of asthmatic patients [105–107]. Finally, increased numbers of mitochondria and changes in mitochondrial ultrastructure and mitochondrial swelling have been shown in the bronchial epithelium of asthmatic mouse models and human asthmatics [108–110]. Mechanisms underlying increased mitochondrial ROS production are unclear; however, reduced expression of glucocorticoid and estrogen receptors in mitochondria of lung epithelial cells in human asthmatics may be involved in reduction in OXPHOS enzyme biosynthesis, mitochondrial impairment, elevated ROS production, and induction of apoptosis in epithelial and other

cell types [10, 111, 112]. Additionally, glucocorticoid and estrogen receptors are involved in regulation of apoptotic, and inflammatory responses [113, 114] and reduced expression of both these receptors could contribute to reduced protection against apoptosis and inflammation in asthma [115]. Interestingly, preexisting mitochondrial dysfunction induced by environmental pollutants intensifies allergic airway inflammation implying that mitochondrial defects could be a risk factor for the development of allergic disorders in susceptible individuals [116]. Although no specific therapy is currently available to minimize mitochondrial dysfunction in experimental models and human asthma, mitochondria-targeted antioxidants may be a promising approach; however, they have yet to be tested as anti-asthmatic drugs.

3. *Pulmonary arterial hypertension:* Pulmonary arterial hypertension (PAH) is a lethal disease of the pulmonary vasculature characterized by pulmonary vasoconstriction, right ventricular hypertrophy, and right ventricular failure [117–119]. The hallmark of PAH is excessive proliferation of pulmonary artery smooth muscle cells that thicken the lumen and increase resistance in pulmonary arteries [120]. PAH is a complex vascular disease, and several environmental and genetic factors including hypoxia, loss-of-function mutations of bone morphogenetic protein receptor II, and viral infections have been linked to the development of PAH [121]; however, the cause and mechanisms of the vascular remodeling in PAH remain undefined. Abnormal mitochondria in pulmonary artery smooth muscle cells may suppress mitochondria-dependent apoptosis and contribute to the vascular remodeling in PAH and recent studies indicate a role for Nogo-B in hypoxia-mediated disruption of mitochondria-endoplasmic reticulum stress in mice and human PAH [122]. Several studies have implicated a role for ROS and RNS derived from Nox proteins and mitochondria in the development of PAH [118]. ROS derived from mitochondria may contribute to mitochondrial dysfunction through two pathways. In the first pathway, excess mitochondrial ROS derived from mitochondrial electron transport chain may promote cellular senescence, necrosis, or apoptosis, leading to endothelial dysfunction and vasculopathy [123]. In the second pathway, mitochondrial dysfunction a reduction in H_2O_2 levels may trigger the pathogenesis of PAH. According to this model, changes in mitochondria result in decreased H_2O_2 production, leading to decreased cellular redox potential causing cellular depolarization, opening of voltage-gated potassium Kv1.512, influx of Ca^{2+}, and vasoconstriction of pulmonary artery SMCs [124]. Several studies also have indicated impaired NO signaling and bioavailability in patients with PAH and in animal models of PAH [125–127]. Although mitochondrial dysfunction and mitochondrial ROS and RNS are implicated in PAH, potential antioxidant strategies specifically targeting mitochondrial ROS as therapy in PAH have been investigated only in animal models, and they need to be extended to humans.
4. *Pulmonary fibrosis:* Pulmonary fibrosis is a lung disease characterized by irreversible destruction of lung architecture, abnormal wound healing, and deposition of extracellular matrix proteins leading to organ dysfunction, disruption of gas exchange, and death from respiratory failure. Lung fibrosis could be idiopathic [128] or arise from exposure to environmental toxins such as fibers,

asbestos, metals, pesticides, chemotherapeutic drugs, viruses, multiwalled carbon nanotubes, and radiotherapy [129, 130]. The underlying mechanisms of idiopathic pulmonary fibrosis (IPF) or environmental agents mediated lung fibrosis are poorly understood. However, at the cellular level, injury to the bronchial and alveolar epithelium, epithelial-mesenchymal transition (EMT), activation of macrophages and proliferation, and transformation of fibroblasts to myofibroblasts may all contribute to pulmonary fibrogenesis [131]. At the molecular level, the fibrogenic responses have been linked to growth factors, ROS, TGF-β, matrix metalloproteinases, bioactive lipids such as sphingosine-1-phosphate (S1P), lysophosphatidic acid (LPA), and its G-protein-coupled receptors acting via specific signaling pathways that modulate fibrogenesis in the lung. There is compelling evidence for the role for ROS, TGF-β, TGF-β-mediated ROS, and ROS-dependent TGF-β activation in the development of pulmonary fibrosis in animal models as well as IPF [132–134]. In animal models of asbestos and bleomycin-induced fibrosis and IPF lungs, there is compelling evidence for oxidative stress with increased production of ROS, which could modify macromolecules and alter cellular functions of epithelial cells and fibroblasts [135]. ROS derived from NOX1, NOX2, and NOX4 has been implicated in the pathogenesis of pulmonary fibrosis [136, 137]; however, recent studies with asbestos fibers suggest that activation of mitochondrial ROS and the resultant damage on lung tissue, as observed in a murine model of asbestosis-induced lung fibrosis, are important [138]. Further, macrophage-derived mitochondrial H_2O_2 seems to play a key role in asbestos fiber-mediated pulmonary fibrosis [138, 139] that was blocked by catalase or knockdown of iron-sulfur protein of complex III in the mitochondrial electron transport chain, a major site of ROS production [140]. Further, accumulating data suggest a potential role for epithelial endoplasmic reticulum stress and mitochondrial ROS in alveolar epithelial cell apoptosis in IPF and asbestos-mediated pulmonary fibrosis [141, 142] (Fig. 1.4). In addition to ROS, dysregulation of TGF-β expression and/or signaling was shown to play an important role in the pathogenesis of pulmonary fibrosis. Interestingly, the interactions between ROS and TGF-β influence the process of fibrogenesis. ROS, derived from NOX and mitochondria, activates latent TGF-β and induces TGF-β expression in many cell types including alveolar epithelial cells and macrophages [143], and conversely, TGF-β also increases ROS production by activating NOX4 [136, 137] and activation of complex III of the mitochondrial electron chain [144]. Mitochondrially targeted antioxidants or genetic disruption of mitochondrial complex III significantly attenuated TGF-β-induced profibrotic gene expression suggesting that targeting mitochondrial ROS may be beneficial in pulmonary fibrosis [144].

5. *Bronchopulmonary dysplasia:* Bronchopulmonary dysplasia (BPD) is one of the devastating complications of premature newborns with no proven therapy. The development of BPD is associated with a number of risk factors including hyperoxia, ventilator-induced lung injury, and pre- and postnatal infections [145]. An arrest of alveolar development, secondary to mitochondrial bioenergetics failure and reduced ATP production, is one of the hallmarks of BPD [146]. Supplemental oxygen therapy (ventilator) resulting in oxygen toxicity, mediated by production and/or accumulation of ROS, has been implicated in the development of BPD in

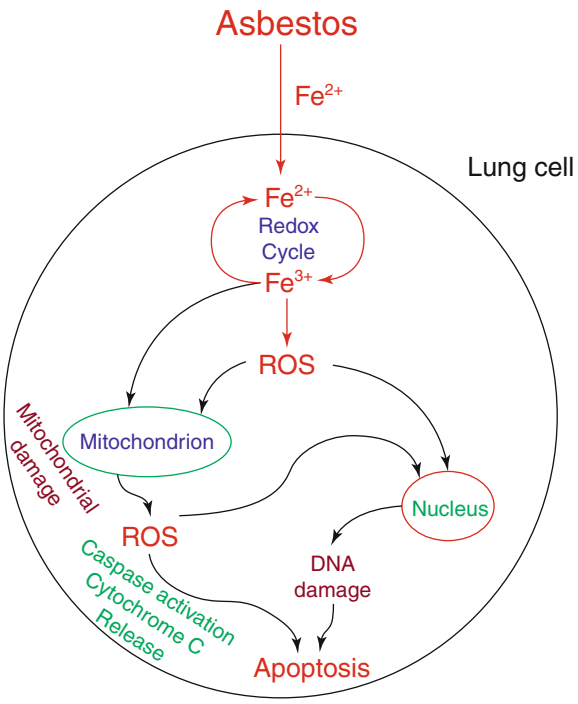

Fig. 1.4 Asbestos-induced ROS generation, mitochondrial damage, and cytotoxicity in lung cells. Asbestos induces cellular ROS generation in lung cells through iron (Fe) redox cycle which cause damage to the mitochondria and nucleus. Asbestos also induces mitochondrial generation of ROS which cause mitochondrial dysfunction and cellular damage including DNA damage, activation of caspases, cytochrome *c* release, and apoptosis

premature infants and experimental models of hyperoxia-induced injury [147, 148]. Hyperoxia generates excessive ROS, which can directly target and injure alveolar epithelium and lung endothelium; however, the source of ROS in hyperoxia-induced lung injury remains controversial. Some of the earlier reports suggest that mitochondria-derived ROS is a critical determinant of hyperoxic cell damage and apoptosis using respiration-deficient cell lines [149, 150]. Additionally, using nine genetically engineered mice deficient in or overexpression of proteins, a role for mitochondrial oxidants initiating BAX- or BAK-dependent alveolar epithelial cell death contributing to hyperoxia-induced lung injury has been demonstrated [151]. This is in contradiction to an earlier report that showed that hyperoxia-induced cell death to be independent of mitochondrial ROS but requires expression of Bax or Bak proteins [152]. Further, hyperoxia increased ROS formation in pulmonary capillary endothelial cells of rat lungs, which was blocked by complex I inhibitor rotenone as well as inhibitors of NADPH oxidase and the intracellular calcium chelator BAPTA [153]. These data suggest a role for both mitochondrial and NOX-derived ROS from the pulmonary endothelium in situ in hyperoxia-induced lung injury. However, exposure of human lung endothelial cells to hyperoxia enhanced ROS generation that was dependent on NOX activation and independent of mitochondrial electron transport chain [154]. There is no clear explanation for the varying observations on the involvement of mitochondrial vs. NOX proteins in hyperoxia-induced ROS generation; however, use of different cell types, variation due to the use of mouse, rat,

and humans, or differential levels of antioxidants in various cell types may all account for the discrepancies. Studies in animal models and premature infants have shown that expression and activity of antioxidant enzymes (AOEs) increase during the third trimester in utero, and premature infants have relative low levels of AOEs [155] that can upset redox balance of the cell leading to apoptosis and inhibition of lung development [151, 156]. Animal studies point out that adult animals are more vulnerable to hyperoxia compared to neonates [157, 158] suggesting reduced cellular antioxidant capacities relative to the newborn animals. Although developmental differences in oxidative stress responses were observed in the murine lung, greater lethality of adult animals exposed to hyperoxia may be due to inflammation rather than to differences in AOEs [159]. The mechanism(s) of increased ROS generation by changes in oxygen pressure from 150 to 300 mm of mercury is unclear, but hyperoxia stimulates signaling pathways mediated by Rac1 [153, 160], MAP kinases [154, 161], PI3 kinase-Akt [162], cytoskeletal redistribution [163], and formation of lipid rafts [164] that contribute to NOX-dependent and NOX-independent ROS formation ultimately leading to lung injury. A recent study suggests the potential involvement of sphingosine-1-phosphate (S1P) and sphingosine kinase (SphK) 1 signaling axis in a murine model of neonatal BPD [165]. Exposure of neonatal mice to hyperoxia enhanced sphingosine-1-phosphate (S1P) levels in lung tissues and *Sphk1*$^{-/-}$, but not *Sphk2*$^{-/-}$ or *Sgpl1*$^{+/-}$, mice offered protection against hyperoxia-induced lung injury with improved alveolarization and alveolar integrity compared to their wild-type counterparts. Further, SphK1 deficiency attenuated hyperoxia-induced accumulation of IL-6 in bronchoalveolar lavage fluids and NADPH oxidase (NOX) 2 and NOX 4 protein expression in lung tissue. In vitro experiments using human lung microvascular endothelial cells (HLMVECs) showed that exogenous S1P stimulated intracellular ROS generation, while transfection with SphK1 siRNA or the use of specific SphK inhibitor attenuated hyperoxia-induced S1P generation. Further, knockdown of NOX2 and NOX4, using specific siRNA, reduced both basal and S1P-induced ROS formation. These results suggest an important role for SphK1-mediated S1P signaling regulated ROS in the development of hyperoxia-induced lung injury in a murine neonatal model of BPD [165]. Further studies are necessary to delineate the therapeutic potential of small molecule inhibitors of sphingosine kinase 1 as well as other enzymes involved in S1P metabolism and antioxidant therapy such as thioredoxin treatment [166] on hyperoxia-mediated alveolar simplification, cell death, and mitochondrial dysfunction.

Conclusion and Future Perspectives

Mitochondria, often referred to "the powerhouses of the cell," play an important role in cellular redox status, signaling, innate immunity, aging, and homeostasis. In addition to producing ATP, the primary cellular energy, transfer of electrons through the mitochondrial electron transport system generates superoxide and subsequently

hydrogen peroxide that are required for normal cell signaling and homeostasis. There is an increasing body of evidence linking mitochondrial ROS to normal cell signaling. Levels of mitochondrial ROS are regulated by antioxidant enzymes and thiols and the quantity of mitochondria in the cell. The expression and activity of antioxidant enzymes and thiol levels in cells are regulated by different mechanisms. Thus, under normal physiological conditions, basal mitochondrial ROS signals to maintain cellular homeostasis. However, under stress or pathological situations, mitochondrial damage and dysfunction occurs, which can be induced by excess of mitochondrial ROS production and/or altered mitochondrial dynamics resulting in altered signaling pathways, modulation of transcriptional factors, immunity, and metabolic adaptation. Increased mitochondria-derived ROS and mitochondrial dysfunction have been implicated in the pathogenesis of a variety of disorders including diabetes, cancer, and lung diseases such as ALI, asthma, pulmonary hypertension, lung cancer, lung fibrosis, and bronchopulmonary dysplasia. Therefore, detoxification of mitochondrial ROS with antioxidants or antioxidant enzymes may be beneficial in ameliorating mitochondrial dysfunction. Indeed, there has been considerable progress in the development of mitochondria-targeted small molecule antioxidants that exhibited reduced mitochondrial oxidative damage and partially prevented decline in mitochondrial function in animal models of lung diseases. However, currently there have been only a few clinical studies to determine the efficacy of antioxidant targeting therapy in human lung pathologies, and there is an urgent need for more phase I and phase II clinical trials. In addition to antioxidant therapy against mitochondrial ROS, it will be critical to identify new and novel signaling pathways dysregulated by mitochondrial ROS as potential targets. An alternate approach would be to reduce accumulation of dysfunctional or damaged mitochondria via modulation of the mitochondrial dynamics (fusion and fission); however, the feasibility of this intervention is yet to be verified in animal models or cell culture models. In addition to drug therapy, interventions such as calorie restriction and exercise have been shown to modulate mitochondrial respiratory chain activity and biogenesis, respectively, that can regulate mitochondrial ROS via eNOS activity [167–169]. Targeting mitochondrial ROS therefore provides a promising platform to launch new therapeutics to combat number of respiratory diseases.

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Chapter 2
Regulation of Mitochondrial Functions by Transcription Factor NRF2

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Abstract Protective and adaptive responses initiated by lung-resident and infiltrated cells play an important role in mitigating the detrimental effects of various toxicants. However, the development of a variety of pulmonary diseases has been attributed to a dysfunctional cellular response following acute or chronic toxicant exposure, resulting from altered gene expression. Although mitochondria have been long thought as cellular powerhouses and regulators of bioenergetics, their biogenesis is promoted by diverse patho-physiological stimuli including cell division, development, exercise, postnatal breathing, metabolism, oxidative stress, and inflammation. Emerging evidence strongly supports the idea that mitochondrial dysfunction caused by various toxicants and pro-oxidants is the origin of pathogenesis and ultimately results in morbidity and mortality. The transcriptional factor nuclear factor (erythroid-derived 2)-like 2 (Nfe2l2 or NRF2), by binding to the antioxidant response element (ARE) of the promoters of redox-sensitive genes, induces the expression of cytoprotective and antioxidative proteins that play a crucial role in mitigating the cellular stress and damage caused by pro-inflammatory and oxidant stimuli. Depending on the extent of its activation, redox signaling can promote either beneficial stress-resolving mitochondrial activity or mitochondrial dysfunction. Accumulating evidence suggests that a deficiency of NRF2 causes mitochondrial dysfunction, culminating in severe lung injury and inflammation. This review

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discusses the biology and role of NRF2 in regulating mitochondrial functions and summarizes current strategies used to target NRF2 in order to confer protection against pulmonary disorders linked to mitochondrial dysfunction.

Keywords Autophagy • Mitophagy • Antioxidants • Lung diseases • KEAP1 • ROS

Abbreviations

AD	Alzheimer's disease
ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
ARE	Antioxidant response element
ATG	Autophagy gene
ATP	Adenosine-5′-triphosphate
BCL	B-cell lymphoma
CDDO-Im	1-[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl] imidazole
CO	Carbon monoxide
COPD	Chronic obstructive pulmonary disease
ETC	Electron transport chain
Fas-L	Fas ligand
GCL	Glutamate cysteine ligase
GCLC	Glutamate cysteine ligase catalytic subunit
GCLM	Glutamate cysteine ligase modifier subunit
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSK3β	Glycogen synthase kinase 3β
GSR	Glutathione reductase
GSSG	Glutathione disulfide
GST	Glutathione transferase
HMOX1	Heme oxygenase1
LPS	Lipopolysaccharide
mGSH	Mitochondrial glutathione
mGSSG	Mitochondrial glutathione disulfide
MOMP	Mitochondrial outer membrane permeabilization
mOXPHOS	Mitochondrial oxidative phosphorylation
mtDNA	Mitochondrial DNA
mtER	Mitochondrial estrogen receptor
mtGR	Mitochondrial glucocorticoid
NQO	NAD(P)H:quinone oxidoreductase
NRF-1	Nuclear respiratory factor-1
NRF-2	Nuclear respiratory factor-2
NRF2	Nuclear factor (erythroid-derived 2)-like 2
Ogg1	8-Oxoguanine DNA glycosylase 1

PD	Parkinson's disease
PGAM5	Mitochondrial phosphoglycerate mutase family member 5
PGC	Peroxisome proliferator-activated receptor gamma coactivator
PHB	Prohibitin
PKB	Protein kinase B
PPAR	Peroxisome proliferator-activated receptor
PRDXs	Peroxiredoxins
RNS	Nitrogen-based reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SRX	Sulfiredoxin
TFAM	Mitochondrial transcription factor A
TNFα	Tumor necrosis factor-alpha
TXN	Thioredoxin
TXNRD	Thioredoxin reductase

Mitochondrial Biogenesis and Oxidative Stress

The mitochondria have long been thought of as simply cellular powerhouses, whose main function is to provide ATP through the oxidative phosphorylation. ATP generation is critically dependent on five intramembrane complexes and two mobile electron carriers, coenzyme Q and cytochrome C [1]. Cytochrome C, an essential component of the electron transport chain (ETC), is located in the outer face of the inner membrane (intermembrane space) of the mitochondrion [2]. Inside the mitochondrion are the cristae, structures that are formed by the folding of the mitochondrial inner membrane and provide increased surface area for chemical reactions to take place within the organelle [3]. The mitochondrial matrix is essential for the processes of fatty acid oxidation, the urea cycle, and the biosynthesis of iron sulfur centers and heme. However, this narrower "metabolic view" of the mitochondria has been transformed over the past two decades as a result of our discovery of new functions for these organelles, such as their obligatory roles in driving intrinsic pathways of apoptosis, oncogenesis, calcium homeostasis, and oxygen sensing [4–6]. Consequently, mitochondria are at the center of many biological responses, and redox signals to and from this organelle help integrate their function with that of the cell and organism. These functions include, but are not limited to, innate immunity [7, 8], differentiation, hormone signaling [9, 10], and determination of life span [11–13]. More importantly, the presence of their own genome, encoding tRNAs, rRNAs, and several mitochondrial proteins, allows mitochondria to operate semiautonomously within the cell, unlike other organelles [14]. That said, the limited coding capacity of mitochondrial DNA (mtDNA) makes this organelle obligatorily dependonence for its molecular architecture on the nuclear genome [15–17]. For example, the lion's share of more than 100 subunits of the respiratory machinery is nuclear genome derived, and a number of mitochondrial import and assembly

factors are encoded by nucleus-derived mRNAs. Finally, the nuclear genome also provides several key factors required for the replication and expression of the mitochondrial genome, including nucleic acid polymerases, RNA processing enzymes, and transcription and replication factors, as well as tRNA synthetases, translation factors, and ribosomal subunits. Thus, it is conceivable that mitochondrial biogenesis, i.e., the formation of new mitochondria, involves the well-orchestrated coordinate actions of nuclear and mitochondrial genes.

Mitochondrial biogenesis is regulated by diverse physiological or pathological stimuli that include, but are not limited to, cell division, development, exercise, postnatal breathing, hormonal secretion, oxidative stress, metabolism, and inflammation [18–21]. Thus, most of the genes required for mitochondrial biogenesis are under the control of a nuclear network of DNA-binding transcription factors and their co-regulators. The primary regulators of mitochondrial biogenesis include the peroxisome proliferator-activated receptor (PPAR) gamma coactivator (PGC) family of transcriptional activators, which consists of PGC-1α, PGC-1β, and PGC-related coactivator (PRC) [22]. PGC-1α not only induces the expression of nuclear respiratory factor-2 (NRF-2), but also together with NRF-2 it stimulates the expression of the related gene NRF-1. Consequently, NRF-1 activates nuclear genes that encode mitochondrial proteins, including the transcription, translation, and repair of mitochondrial transcription factor A (TFAM), TFB1M, and TFB2M, which are essential for controlling mtDNA [18, 19, 23].

Reactive oxygen species (ROS) are a family of highly oxidizing short-lived molecules containing oxygen (e.g., oxygen ions and peroxides). The related, but chemically distinct, reactive nitrogen species (RNS) are also essential players in cell signaling. Although RNS are implicated in both mitochondrial functions and dysfunction, this review mainly will focus on how ROS production is regulated and how it contributes to mitochondrial function and dysfunction. The ROS are generated from several exogenous and endogenous sources [24]. Exogenous sources of ROS include UV and visible light, ionizing radiation, drugs, and environmental toxins; the endogenous sources include xanthine oxidase, cytochrome P-450 enzymes in the endoplasmic reticulum, peroxisomal flavin oxidases, and plasma membrane-associated NADPH oxidases. Although, the major endogenous source of ROS is the mitochondrial electron transport chain, other enzyme systems in the mitochondria can also contribute [25]. Under physiological conditions, ROS levels are kept low by scavenging enzymes, and, thus, a "redox balance" is achieved in the cell [26]. Redox balance, the ratio between oxidizing and reducing species, is involved in the regulation of various signaling pathways, including the activity of protein kinases and phosphatases, which through the modulation of the post-translational modifications of certain transcription factors regulates gene expression [27, 28]. Excessive ROS production or defective activation of antioxidant defenses results in oxidative damage and pathological conditions. ROS not only damage DNA, proteins, and fatty acids by direct oxidation, but also activate specific signal transduction molecules that affect cell survival.

Various physiological processes are dependent on cellular detoxification to maintain the intracellular redox status. In healthy cells, the continuous production of

ROS is balanced by scavenging reactions operated by various antioxidant enzymes. The antioxidant enzymes or molecules that inactivate ROS include glutathione transferases (GSTs), glutathione peroxidases (GPXs), glutathione reductases (GSRs), superoxide dismutases (SODs), NAD(P)H:quinone oxidoreductases (NQOs), thioredoxins (TXNs) and thioredoxin reductases (TXNRDs), catalase, heme oxygenases (HMOXs), peroxiredoxins (PRXs) and sulfiredoxins (SRXs), and glutamate cysteine ligase (GCL) catalytic (GCLC) and modulatory (GCLM) subunits required for the biosynthesis of glutathione (GSH), a major cellular antioxidant. Other molecules such as vitamin E, vitamin C, metallothioneins, and heat shock proteins are known to modulate cellular stress and provide cellular protection against various oxidant- or pro-oxidant insults [29–31]. Data obtained from cell systems and experimental models of human diseases and by using genetic and pharmacological approaches have demonstrated protective roles for several of the antioxidant enzymes and proteins described above in preventing and mitigating tissue injury and inflammation [32, 33]. Several lines of evidence suggest that the progression of lung diseases is most likely the result of a dysfunctional cellular antioxidant defense system or of certain genetic defects that deregulate host factors elicited by pro-oxidant or toxicant exposure [34, 35].

Mitochondrial Apoptosis and Autophagy

Cellular homeostasis is achieved through the renewal of essential macromolecules to preserve the proper functioning of organelles such as mitochondria. Mitochondria predominantly synthesize ATP, which is critical for maintaining bioenergetic homeostasis; however, they are also major components of a canonical cell death pathway, apoptosis. Apoptosis governs the development of organs, as well as the immune response, cell survival, and tissue homeostasis. Two major pathways of apoptosis, extrinsic and intrinsic, control cell death [36]. The extrinsic pathway requires the engagement of cell death-inducing ligands, e.g., TNFα and Fas-L, with their cell-surface receptors, resulting in the autocatalytic activation of cysteine proteases of the caspase family. These proteases, in turn, cleave various cellular proteins to execute a cell death. The intrinsic pathway relies on the opening and closing of the mitochondrial outer membrane permeabilization (MOMP) transition pores, which release death-activating proteins into the cytoplasm [37, 38]. For example, the release of cytochrome C from the mitochondrion activates caspase-9 in association with a scaffolding protein, the apoptosis-activating factor 1. MOMP is controlled by several nucleus-encoded proteins belonging to the B-cell lymphoma (BCL) family, whose founding member is the BCL2 protein [39]. Two major subtypes of the BCL family, the anti- and pro-apoptotic classes, regulate MOMP. The antiapoptotic subtype is typified by the BCL2 and BCL-X_L proteins, which close MOMP pores, and the pro-apoptotic by BAX and BID (a membrane-targeted death ligand), which open them. The BID protein is a unique member of this family, since it couples the extrinsic and intrinsic pathways. BID exists as a dormant pro-apoptogenic protein in the

cytoplasm, and it is cleaved by death receptor-activated caspase-8 at a specific site to generate a truncated form (t-BID), which then opens the MOMP pores [40]. The opening of the pores amplifies the extrinsic pathway signals by activating the intrinsic pathways. As already mentioned, caspases are essential players in apoptosis. Their active sites are subject to redox regulation. For example, TXN and TXNRD, are critical for executing cell death in response to interferons [41, 42]. Redox-active TXN is necessary for keeping caspase active sites in a reduced state. Consistent with this effect, nitrosylation of caspases suppresses their biological activity [43–45].

Autophagy is, by definition, the self-eating of a cell. It serves both as a growth-promoting and growth-suppressing pathway in mammals. Three forms of autophagy, chaperone assisted, micro, and macro, are known to occur in mammalian cells [46]. Macroautophagy is a well-orchestrated process, in which multiple gene products participate. In autophagy, damaged organelles or invading pathogens are isolated by an intracellular double membrane, which is derived from the endoplasmic reticulum (Fig. 2.1). Depending on the organelle being degraded, autophagy is also referred to as mitophagy, ERphagy, and pexophagy [47]. Starting at specific points, the initial membranes (known as autophagophores) progressively grow around the target until they coalesce. The formation of this final structure, known as an autophagosome, depends on dozens of proteins called autophagy gene (ATG) products. Subsequently, autophagosomes fuse with lysosomes, and the contents of the autophagosomes are digested and the resulting amino acids, sugars, and fatty acids sent back into cytoplasm, where they enter biosynthetic pathways. Although autophagy is not a substitute for a proteasome-related degradation (which degrades damaged, short-lived, or improperly folded proteins in an ubiquitylation-dependent manner), it degrades aggregated, damaged proteins under conditions of proteasome failure. p62/sequestosome 1 (SQSTM1) binds to poly-ubiquitylated proteins, forming a structure called a "sequestosome," and delivers the cargo to the autophagosome for destruction [48]. Thus, autophagy maintains homeostasis by functioning as an "intracellular recycling system" for the cell, maintaining a pool of organelles that are performing well and removing unfit and damaged ones [49, 50] (see Fig. 2.1). Autophagy also provides a major mechanism for intracellular organelle turnover and assists in host defense and innate immune responses by degrading the invading pathogens and/or by dampening inflammatory cascades [47].

Mitophagy targets depolarized mitochondria, notably during apoptosis. It results in the formation of an isolation membrane enclosing organelles and fragments of the cytoplasm that are delivered into lysosomes for hydrolytic digestion and recycling. Mitophagy ensures that damaged or excessive mitochondria are properly directed toward degradation via autophagy [47]. Thus, autophagy is important for recycling intracellular components for cell survival and also essential for maintaining organelle homeostasis and quality. Not only is autophagy important for mitochondrial function, but also there is growing body of evidence that the mitochondrion itself plays a significant role in autophagy. For example, mitochondrial dysfunction has been associated with neurodegenerative disease such as Parkinson's and also with some metabolic disorders [51]. The mitochondria and the process of autophagy are uniquely related, in that defects in either can increase the risk of certain

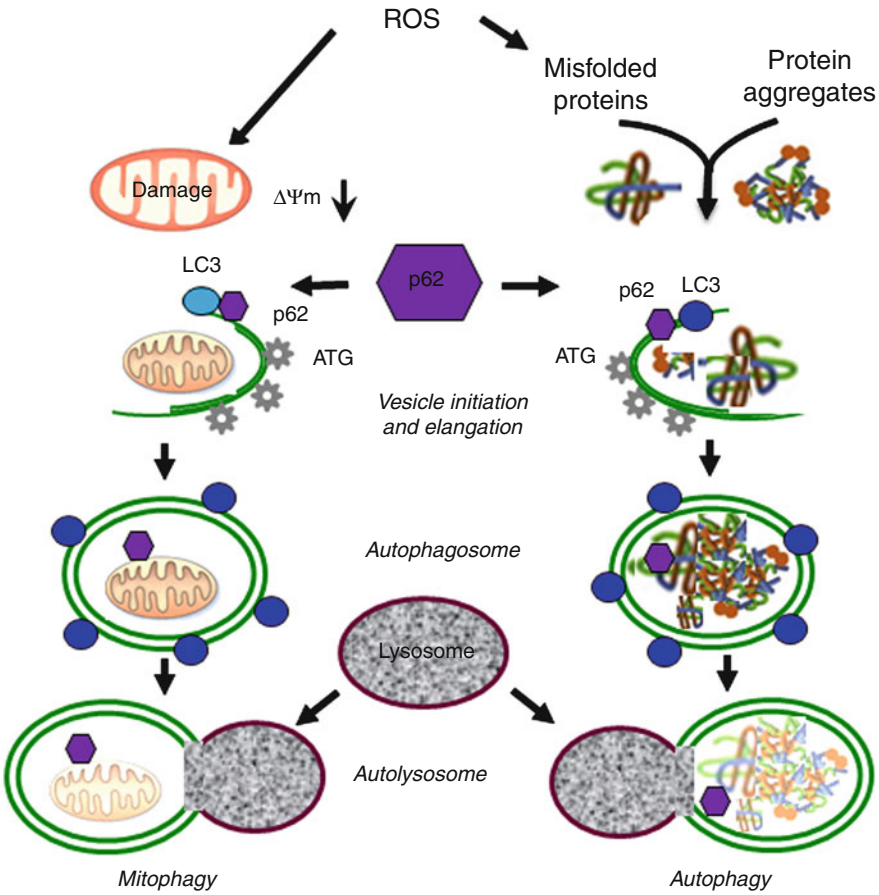

Fig. 2.1 Regulation of mitophagy and autophagy. Schematic summarizes the p62/SQSTM1-regulated formation of autophagosome for recycling the damaged mitochondria and misfolded and aggregated proteins. Autophagy and mitophagy proceed through several phages including initiation, elongation, maturation, cargo sequestration, and autophagosome and lysosome fusion. p62 recruits LC3 to the autophagosome membranes that is required for the autophagosome maturation

metabolic and autophagic diseases [52]. Further investigations of the functional interactions between mitochondrial and autophagy functions and dysfunctions would reveal the mechanisms underlying pathogenesis of various diseases.

Mitochondrial Dysfunction in Lung Diseases

Mitochondria act as sensors of oxidative stress and a focal point of cellular signaling platforms, especially those involved in modulating cell death, including necrosis, apoptosis, and autophagy [53–57]. A deficiency in energy metabolism, the

bioenergetic failure characteristic of mitochondrial disease states [58], has been implicated in a variety of human diseases. Various diseases, such as glaucoma, inflammation, neurodegenerative diseases, type 2 diabetes, cardiomyopathies, dysrhythmias, and cancers, especially those involving prostate and colon, have been linked to mitochondrial dysfunction (reviewed in [59]). All these diseases have been associated with defects in mitochondrial function [60–63] or with an inability to accommodate the consequences of oxidative stress [64]. Although ROS act as signaling molecules at low/physiological levels, excessive ROS production causes nuclear DNA damage and the degradation of oxidized proteins, lipids, and nucleic acids. It also inflicts damage on various cellular organelles, particularly mitochondria [65]. The spatial proximity of mtDNA to the free radicals produced by the electron transport chain (ETC) makes it uniquely susceptible to mutations, especially when the ETC becomes dysfunctional [66]. The current view is that ROS are a primary cause of mitochondrion-driven diseases and mitochondrial dysfunction, and the resulting oxidative damage can have profound effects, such as a buildup of cytotoxic metabolites, energy depletion, and even cell death and organ dysfunction [12, 67].

During development, in high-altitude travel, and in acute and chronic lung disease states, lung cells are exposed to hypoxia, and the release of ROS from the inner mitochondrial membrane is triggered by hypoxic conditions. The ETC serves as the critical cellular oxygen sensor for many of these responses. Although ROS signals generated by hypoxia are thought to play a crucial role in pulmonary development in the newborn and produce responses in mature lungs (primarily guarding cells from hypoxic injury), unremitting ROS production and signaling are counterproductive, because they inflict cellular damage and degrade the normal function of the lungs [68]. The lungs of newborns and adults are constantly exposed to various environmental stressors that cause epithelial and endothelial cell dysfunction and death, leading to acute lung injury and inflammation and respiratory impairment. A prominent role has been suggested for mitochondrial dysfunction in acute and chronic lung disorders. For example, damage to mitochondria was observed in the lungs of rats subjected to ischemic reperfusion, and this damage was accompanied by tissue injury and inflammation [69]. Blocking mitochondrial complex III activity reduces LPS-induced, ROS-mediated acute lung injury, pointing to a central role for mitochondrial ROS in promoting pulmonary disorders [70]. Exposure of mice to LPS causes oxidative mitochondrial damage and biogenesis in cardiomyocytes, and LPS-induced lung injury is accompanied by mitochondrial biogenesis, suggesting that this process is important for tissue repair and resolution [71]. Decreased levels of mitochondrial glucocorticoid (mtGR), estrogen (mtER) receptors, and mitochondrial oxidative phosphorylation (OXPHOS) enzyme biosynthesis have been observed in the lungs of mice with allergic airway inflammation, suggesting that either a loss or decreased function of these proteins contributes to airway diseases such as asthma [72]. Genetic mutations in SARS2, which encodes mitochondrial seryl-tRNA synthetase, have been found in infants with hyperuricemia, pulmonary hypertension, renal failure, and alkalosis (HUPRA) syndrome and are accompanied by a lack of acylated tRNA [73].

Prohibitins (PHB1 and PHB2) form large, multimeric ring complexes in the inner membrane of mitochondria and interact with the NADH dehydrogenase protein complex, constituting an essential pathway for the mitochondria [74]. Knockdown of PHB1 or PHB2 leads to mitochondrial damage and dysfunction, accompanied by enhanced levels of ROS generation in adipocytes [75]. Prohibitin expression was shown to be downregulated in the lungs of COPD and non-COPD smokers, suggestive of an alteration of mitochondrial function, possibly because of decreased mitochondrial stability caused by cigarette smoke exposure. This downregulation may be causally linked to genesis of COPD [76]. Further studies are warranted to better define the exact mechanisms of regulation and the functions of nuclear and mitochondrial genes encoding proteins that control mitochondrial functions in the lungs during development and to characterize the physiological conditions and pathological states caused by environmental stressors.

Several studies have explored the use of small molecules, peptides, and proteins that target mitochondrial ROS as part of a strategy to mitigate cellular stress and tissue damage, and they have displayed beneficial effects in culture systems and animal models of lung diseases. For example, oxidant stress causes mtDNA damage and death in pulmonary artery endothelial cells, but overexpression of a DNA repair enzyme, Ogg1, mitigates mtDNA damage and cytotoxicity [77]. Pretreatment of rodents with antioxidants suppresses oxidant-induced lung pathogenesis, but their use to improve the outcomes of patients in the clinical setting has had only limited beneficial effects, if any [78–80]. Nevertheless, new and novel approaches are being evaluated to improve human health, with specific emphasis on enhancing mitochondrial functions. For example, a new study using an LPS-induced model of acute lung injury has demonstrated that bone marrow-derived stromal cells can repair tissue injury through the transfer of mitochondria [81]. This mitochondrial transfer resulted in increased levels of alveolar ATP concentrations, suggesting that mitochondrial transfer can rescue and repair injured cells, a finding that could have important implications for a variety of diseases linked to abnormal tissue repair [81].

Regulation of Mitochondrial Metabolism and Functions by the NRF2-ARE Pathway

NRF2 is a cap'n'collar basic leucine zipper transcription factor. The Kelch-like ECH-associated protein 1 (KEAP1) retains NRF2 in the cytoplasm and promotes its proteasomal degradation [82]. Several stressful and electrophilic stimuli are known to disrupt KEAP1/NRF2 interactions, leading to the release of NRF2 from the cytoplasm and its subsequent nuclear accumulation [83–85]. In the nucleus, NRF2 heterodimerizes mainly with the MAF (Maf-G, Maf-F, and Maf-K), JUN (c-Jun, Jun-B, and Jun-D), and ATF (ATF-4) families of bZIP proteins prior to binding to the DNA sequence 5′-TGAG/CnnnGC-3′ (the antioxidant response element (ARE)), resulting in the transactivation of a network of genes that encode cytoprotective and

Table 2.1 NRF2-regulated mitochondrial proteins encoded by nuclear DNA

Gene name	Gene symbol	References
Aldo-keto reductase	AKR1B	Nishinaka et al. [87]
Cystine-glutamate transporter	xCT	Sasaki et al. [88]
Glutathione S-transferase alpha 3	GSTα3	Tjalkens et al. [89]
Glutathione S-transferase alpha 4	GSTα4	Hayes et al. [90]
Glutathione S-transferase mu1	GSTmu1	Tjalkens et al. [89]
Glutathione S-transferase pi1	GSTpi1	Ikeda et al. [91]
Glyoxalase 1	GLO1	Xue et al. [92]
Malic enzyme 1	ME1	Mitsuishi et al. [93]
Isocitrate dehydrogenase 1	IDH1	Mitsuishi et al. [93]
Nuclear respiratory factor 1	NRF-1	Piantadosi et al. [94]
Peroxiredoxin 1	PRDX1	Kim et al. [95]
Peroxiredoxin 3	PRDX3	Miyamoto et al. [96]
Peroxiredoxin 5	PRDX5	Miyamoto et al. [96]
Sulfiredoxin	SRX	Soriano et al. [97]
Superoxide dismutase 1	SOD1	Dreger et al. [98]
Thioredoxin	TRX1	Kin et al. [99]
Thioredoxin reductase 1	TXNRD1	Sakurai et al. [100]

antioxidative enzymes and proteins [83, 86]. Some of the well-characterized targets include GPX2, NQO1, GCLC, and GCLM, HMOX1. Further, NRF2 also regulates the expression of nuclear genes that encode several mitochondrial proteins (see Table 2.1) [87–100]. As described above, nuclear respiratory transcription factor NRF-1 regulates mitochondrial gene expression by inducing TFAM.

The promoter of NRF-1 has AREs, the binding sites for NRF2 [94]. Activation of NRF2 enhances mitochondrial biogenesis through transcriptional activation of the NRF-1 promoter [94] (Fig. 2.2). Piantadosi et al. have demonstrated that carbon monoxide (CO), a product of HMOX1 activity, elevates the mtDNA copy number in an NRF2-dependent manner in the lungs of mice subjected to pneumonia [94]. This group has shown that CO elevates mitochondrial H_2O_2 production, especially which in turn activates the PKB/AKT kinase. AKT then phosphorylates (and thus inactivates) GSK-3β, allowing NRF2 nuclear accumulation and potentiation of NRF2-depenent gene expression [101]. By inducing mitochondrial autophagy, especially when the damage to mitochondria caused by oxidative stress is irreparable, HMOX1 confers protection to lung epithelial cells exposed to chronic levels of hyperoxic stress, perhaps by decreasing the abnormal levels of ROS generated by damaged mitochondria [102]. The NRF2-ARE pathway therefore plays a key role in guarding mitochondria against oxidative stress. Recent evidence shows that mitochondrial ROS activate downstream protective mechanisms including the NRF2-ARE pathway [103, 104]. Mitochondrial phosphoglycerate mutase family member 5 (PGAM5), a protein phosphatase, plays an important role in regulating mitochondrial functions. When targeted to the outer membrane of mitochondria, forms a

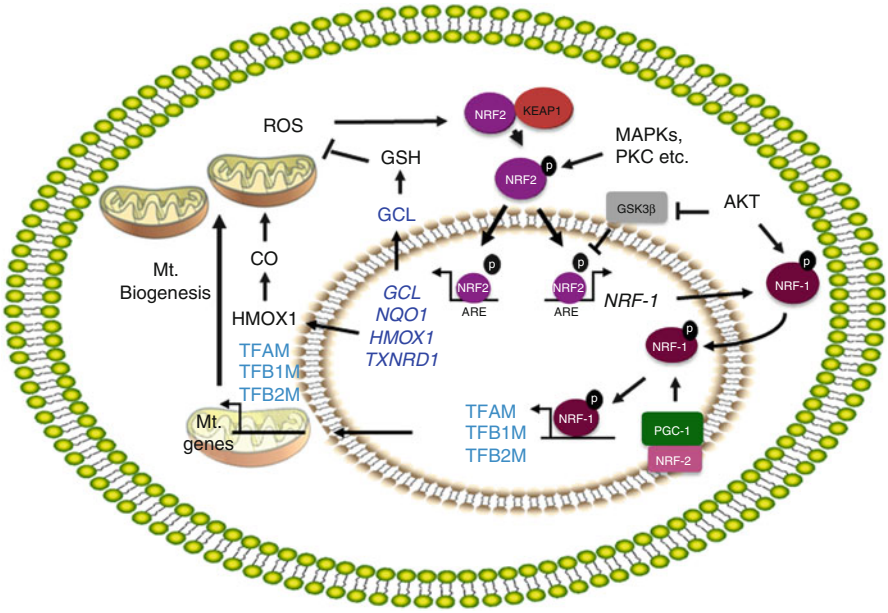

Fig. 2.2 Regulation of mitochondrial biogenesis by N2. Schema summarizes the signaling- and NRF2-regulated proteins involved in countering the ROS-mediated stress and mitochondrial (*Mt*) biogenesis. HMOX1 generates CO by degrading heme, and CO stimulates ROS generation in mitochondria. ROS oxidize KEAP1 cysteine residues and release NRF2 from KEAP1 and phosphorylated by MAP kinases/protein kinase C. NRF2 then translocates into the nucleus and transcriptionally activates the genes that encode antioxidant proteins and NRF-1. NRF-1 upon activation by AKT translocates into the nucleus and further activated by PGC-1 family members and NRF-2. NRF-1 activates several nuclear-encoded mitochondrial genes including mitochondrial transcriptional factors, TFAM, TFB1M, and TFB2M and promotes mitochondrial biogenesis. The *arrows* represent activation, and "*blunted*" *arrows* represent inhibition

ternary complex with the KEAP1-NRF2 dimer [105], and knockdown of either KEAP1 or PGAM5 activates NRF2-dependent antioxidant gene expression in HeLa cells, suggesting that PGAM5 regulates mitochondrial functions by modulating NRF2 activity indirectly via KEAP1[105]. KEAP1 via PGAM5 promotes degradation of antiapoptotic proteins BCL-Xʟ and BCL2 [106], whereas NRF2 upregulates antiapoptotic protein BCL2 [107]. Thus, PGAM5/KEAP1/NRF2 interactions add another dimension to NRF2 regulation and functions during cellular injury and death, because PGAM5 acts as a crucial player in promoting necrotic cell death [108]. It would be important to determine under what conditions the KEAP1-NRF2 complex tethered to mitochondrial membrane by PGAM5, the relevance of PGAM5/KEAP1/NRF2 interactions during stressful insults, and how oxidative stress perturbs these interactions favoring mitochondrial dysfunction and autophagy or cell death in response to chronic stress and in disease states (Fig. 2.3).

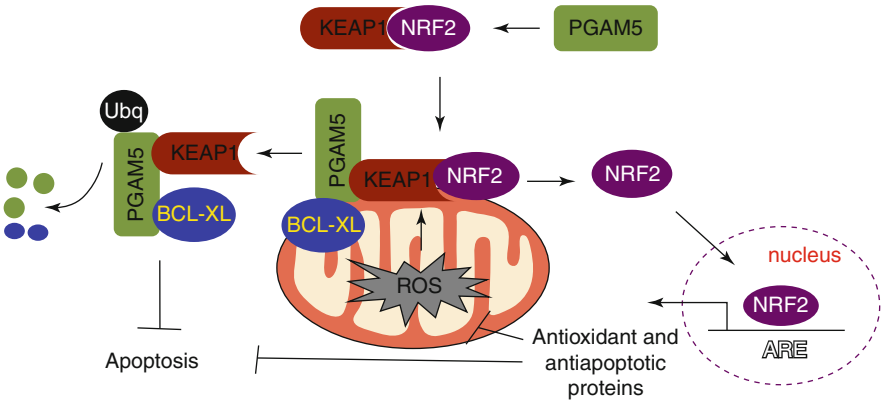

Fig. 2.3 PGAM5-regulated KEAP1-NRF2 interactions and antioxidant response. PGAM5 tethers KEAP1/NRF2 complex to the mitochondrial membrane during mitochondrial stress. Mitochondrial ROS cause dissociation of NRF2 from KEAP1. NRF2 enters into the nucleus and activates antioxidant and antiapoptotic gene expression to mitigate mitochondrial stress. KEAP1 targets PGAM5 and BCL-XL proteins to proteasomal degradation and inhibits apoptosis

NRF2-Regulated GSH Signaling in Mitochondrial Metabolism and Function

NRF2-regulated transcriptional activation of *GCLC* and *GCLM* is crucial for de novo synthesis of GSH, the most abundant nonprotein thiol in the cell, in both constitutive and induced states. Oxidative and electrophilic stress induces de novo GSH synthesis via NRF2-mediated upregulation of the xCT anionic amino acid transporter [109]. Thus, NRF2 plays key roles in mitochondrial metabolism and protection by regulating the expression levels of genes that regulate GSH biosynthesis (*GCLC*, *GCLM*, and xCT or SCLA711) and turnover (see below). Under normal conditions, GSH is synthesized in the cytosol and then relatively slowly transported into the mitochondria [110, 111]. The mitochondrial GSH (mGSH) pool is maintained at 1–5 mM, even when the cytosolic redox balance is disturbed [112, 113]. This concentration is regulated by the action of glutathione reductase (GR) [114, 115], which reduces mGSSG to mGSH using NADPH. GPx, in the presence of GSH, detoxifies H_2O_2, leading to the generation of H_2O and GSSG. There are two forms of GPx in the matrix: Soluble GPx1 [116] mainly degrades H_2O_2, whereas GPx4, located on the matrix surface of the inner membrane, degrades phospholipid hydroperoxides and organic hydroperoxides to alcohols [117–119]. GR then converts GSSG to GSH at the expense of NADPH in order to prevent the loss of GSH. The GSH/GSSG redox couple is an example of a major thiol/disulfide couple in the cell that helps to maintain the overall redox state. Studies have found the GSH/GSSG redox potential inside the mitochondria to be slightly more negative (approximately −280 mV) than the cytoplasm, indicating a more reduced environment [120, 121].

However, much remains unclear about the nature and regulation of mGSH transport and the means by which mitochondria maintain GSH in a reduced form.

The reversible S-glutathionylation of mitochondrial enzymes has emerged as an important mechanism for the regulation of metabolism in response to changes in redox environment and ROS production [122]. The mitochondrial matrix environment, with its basic (pH ~8) conditions and high concentration of GSH [122, 123], provides the necessary milieu to promote thiol residue modification of proteins by GSH. This process has been suggested to act as a protective mechanism to prevent protein oxidation/deactivation, and it also serves as a negative regulator of ROS production from complex I [124]. Glutathionylation also plays a protective role after oxidative stress or ischemia preconditioning because it protects exposed cysteines from oxidative damage. During oxidative stress, the thiols of several proteins undergo reversible oxidation to sulfinic (SO_2H) or sulfonic acid (SO_3H) [125–127]. Hence, by reversibly modifying critical thiols, glutathionylation can protect enzymes in the mitochondria during oxidative stress.

Glutaredoxins (GRXs) catalyze the deglutathionylation of protein-GSH mixed disulfides far more effectively than do other thiol proteins such as thioredoxin (TRX) [128]. Mitochondrial GRX2 has a CSYC motif in its active site, with cysteine 70 playing a key role in deglutathionylation [129–131]. GRX1 has an additional exposed cysteine that is readily modified by oxidants; however, GRX2 lacks this cysteine residue, which may make it less easily inactivated by oxidative stress, S-nitrosating agents, and GSSG within the mitochondrial matrix [129, 131, 132]. Furthermore, GRX2 can be reduced directly by thioredoxin reductase 2 (TXNRD2) [132, 133]; however, this reduction is far less efficient than reduction by GSH, which is more effective even at a GSH level that is 10 % of normal [133]; consequently, the TXNRD2 is unlikely to play a physiologically important role in GRX2 reduction. Together, these factors appear to enable GRX2 to operate more effectively than GRX1 in an environment with more oxidative stress. GRX2 can form an inactive dimer around an iron sulfur center, and this dimer formation can be reversed by oxidative stress, potentially enabling the activation of GRX2 in response to elevated mitochondrial oxidative stress [134–136]. Recently, it has been demonstrated that glutathionylation and deglutathionylation play a key role in controlling ROS-induced proton leakage through uncoupling proteins, with glutathionylation decreasing proton leak-dependent oxygen consumption in mitochondria isolated from skeletal muscle [137]. It would be of great interest to determine the exact relevance and mechanisms of glutathionylation and deglutathionylation balance during mitochondrial functions and dysfunction in acute and chronic lung disease states.

NRF2 Impairment and Mitochondrial Dysfunction

Levels of mitochondrial ROS are counterbalanced by NRF2-regulated antioxidant enzymes and proteins. Some of these enzymes and proteins include HO1 [138], NQO1 [139], GST [140], solute carrier family 7 (SLC7A11) [141], GSH [142],

TXNRD1 [100], and PRDX [141]. Recent studies have demonstrated that NRF2 also regulates expression of the genes encoding proteins that control cellular anabolic metabolism [93]. Dysregulation of the expression of these antioxidative and metabolic enzymes and proteins promotes enhanced oxidative stress, mitochondrial damage, and impairment of mitochondrial function, leading to lung disease states. Consistent with this view, several studies (including ours) using experimental models of stress-induced pulmonary disorders that are known to cause mitochondrial dysfunction have shown that NRF2 confers protection against endotoxin-induced lung injury and septic shock [143, 144], pro-oxidant-induced lung injury and fibrosis [145, 146], and cigarette smoke-induced emphysema [147]. Loss of NRF2 causes a redox imbalance primarily as the result of a diminished or low level expression of genes encoding several antioxidative and cytoprotective enzymes in both the constitutive (basal) states and in response to stressful stimuli in lung-resident cells and alveolar inflammatory cells [83, 86, 148, 149]. Loss of NRF2 also impairs the resolution of sublethal hyperoxic lung injury and inflammation, but supplementation of Nrf2-null mice with GSH following lung injury can improve the resolution of their lung damage [150]. Nrf2-deficient pups, when exposed to hyperoxia at birth for four days, develop greater levels of alveolar simplification (septal growth arrest) than do Nrf2-sufficient pups [151]. Nrf2 deficiency enhances cellular stress and susceptibility to oxidant-induced lung epithelial cell death [152], and its overexpression confers cellular protection against hyperoxia in lung epithelial cells [148, 153], as well against pro-apoptotic stimuli in non-lung epithelial cells [85, 154]. This experimental evidence strongly supports an important role for the NRF2-driven transcriptional response in mitigating cellular stress and mitochondrial functions induced by pro-oxidants and toxicants.

In agreement with the experimental studies described above, a decline in NRF2-regulated cytoprotective gene expression has also been observed in the lungs of patients with COPD, and this loss is associated with the decreased expression of DJ-1 [155], which is known to confer protection against oxidative stress by regulating mitochondrial ROS and functions [156]. An association between NRF2 polymorphisms and an increased risk of susceptibility to acute lung injury (ALI) and decline of lung function in smokers has been reported [157–159]. Pharmacological activation of NRF2 improves the antibacterial defenses of alveolar macrophages in patients with COPD [160] and restores corticosteroid responses in patients with COPD [161], suggesting the involvement of a dysfunctional NRF2 or an inactivation of NRF2 signaling in bacterial exacerbations in smokers. The levels of NRF2 signaling and NRF2-dependent genes are altered in patients with mitochondrial diseases [162], suggesting that decreased NRF2 activity is associated with the progression of mitochondrial diseases. Nuclear translocation of NRF2 has been shown to be impaired in neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's disease (PD), and dysfunction in the NRF2 pathway has been shown to lead to a decreased cellular defense against oxidative stress [163]. In certain disease states such as diabetes, mitochondrial GSH levels are decreased [112, 113, 164]; GSH depletion decreases mitochondrial antioxidant defenses in diabetic cardiomyocytes and renders them more sensitive to apoptosis after an oxidant insult [112]. These findings may explain

why the elderly with diminished NRF2 activity [165] and endogenous antioxidant defenses have a higher incidence of type 2 diabetes and cardiovascular problems. Thus, targeting NRF2 can be a novel therapeutic approach to addressing multifaceted antioxidative and innate responses in order to counteract oxidative stress-induced and mitochondria-mediated lung disorders and other disease states.

Activation of the NRF2 Pathway as a Therapeutic Approach to ameliorate Mitochondrial Dysfunction

Several compounds that specifically disrupt KEAP1/NRF2 interactions, such as isothiocyanates, sulforaphane, indoles, and triterpenoids, confer protection against ALI and inflammation in preclinical models of human disorders, and this protective response has been shown to be correlated with an elevated level of antioxidant gene expression regulated by NRF2 [166, 167]. Notably, triterpenoid analogs are more potent than isothiocyanates and sulforaphane in inducing the NRF2-ARE-mediated transcriptional response, and these compounds have been shown to have beneficial effects at nanomolar concentrations in various preclinical models of tissue injury and inflammation (see review [168]). For example, mice administered with the triterpenoid compound CDDO-Im (1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole) show decreased levels of hyperoxia- [169] and LPS-induced lung injury and inflammation in mice [170], as well as reduced cigarette smoke-induced emphysema [171], when compared to their vehicle-treated counterparts. This protection by CDDO-Im was observed in Nrf2-sufficient but not Nrf2-deficient mice, suggesting that specific targeting of NRF2-ARE signaling may provide a novel therapeutic strategy for treating human diseases. While triterpenoids have shown significant positive outcomes in various preclinical models, a recent clinical study with bardoxolone, an CDDO analog, showed adverse cardiovascular effects in phase III trial of diabetics with severe stage 4 chronic kidney disease, although it improved kidney function [172]. Further refinement of existing NRF2 activators and/or the development of novel activators are clearly needed in order to enhance NRF2-mediated mitochondrial biogenesis and function and improve disease outcomes.

Summary and Future Perspectives

The mitochondria-dependent release of ROS is essential for various physiological responses, such as adaptation to cell growth conditions and variations in oxygen levels. Elevated levels of mitochondrial ROS generated in response to environmental stress, genetic, and metabolic state feedback are known to cause mitochondrial dysfunction, resulting in organ dysfunction and ultimately a pathological state. NRF2-driven ARE-mediated transcriptional responses are crucial for maintaining

intracellular redox homeostasis, and an NRF2 deficiency increases susceptibility to the development of lung pathogenesis in experimental animals. Consistent with these experimental data, NRF2/*ARE* dysfunction has been reported in human lung diseases, including COPD and pulmonary disorders. NRF2 deficiency impairs the resolution of lung inflammation and tissue repair and increases susceptibility to pathogenic infections following exposure to oxidants. Mitochondrial dysfunction has been implicated in all these processes. Recent studies have revealed that NRF2 regulates mitochondrial biogenesis by inducing the gene coding TFAM, a transcription factor that binds to mitochondrial gene promoters and induces their expression, and by upregulating *HMOX1* expression. Because oxidative stress caused by mitochondrial dysfunction can lead to NRF2 activation, mitochondria damage, and mitophagy, it is likely that NRF2 activation acts as an autoregulatory feed-forward loop to dampen the increased ROS levels and to promote mitochondrial biogenesis, thereby maintaining homeostasis following tissue or cellular injury. Recent studies have shown that NRF2 promotes autophagy, indicating that NRF2 modulates mitophagy, rather than mitochondrial biogenesis, under conditions of oxidative damage to the mitochondria. It is unclear whether the impairment of NRF2-ARE signaling contributes to the subsequent pathogenesis caused by mitochondrial dysfunction and likewise whether mitochondrial dysfunction compromises NRF2-ARE signaling and leads to pulmonary disease. Compounds that target NRF2-ARE signaling have shown promising results in experimental models of ALI/ARDS, septic shock, and emphysema. The use of new small-molecule activators that specifically target and upregulate the NRF2-ARE transcriptional response may be a better therapeutic option for blocking cellular stress and promoting mitochondrial biogenesis in diseases linked to mitochondrial dysfunction. Indeed, compounds that target NRF2-ARE signaling are in phase III clinic trails to improve clinical outcomes in patients with various diseases, such as COPD. Although the results are promising, whether these compounds provide a significant improvement in clinical outcomes in critically ill patients remains to be seen.

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Chapter 3
Mitochondrion: A Missing Link in Asthma Pathogenesis

Ulaganathan Mabalirajan, Anurag Agrawal, and Balaram Ghosh

Abstract Asthma is a multifactorial airway disease with airway hyperresponsiveness, airway inflammation, goblet cell metaplasia, and structural changes including airway smooth muscle proliferation and subepithelial fibrosis. Airway epithelial injury and apoptosis is an important triggering and amplification point in asthma pathogenesis, and mitochondrial dysfunction in epithelial cells appears to play an important role. On the other hand, mitochondrial biogenesis is an important aspect of smooth muscle hypertrophy and fibroblast proliferation, which leads to airway remodeling and hyperresponsiveness. In mice, preexisting mitochondrial dysfunction has been shown to potentiate allergic experimental asthma. In this review, we summarize the current understanding on the involvement of mitochondria in asthma pathogenesis, discuss the probable points of intersection between lung pathobiology and mitochondrial biology, and speculate regarding the road ahead. Mitochondrial influence on cellular oxidative and nitrative stress, apoptosis, and calcium homeostasis is covered in detail, as well as the role of molecules like nitric oxide synthase, asymmetric dimethyl arginine (ADMA), and peroxynitrite on mitochondrial function, epithelial injury, and asthma. Potential therapeutic strategies involving coenzyme Q, vitamin E, and esculetin that influence mitochondrial function and alleviate features of asthma are also discussed.

Keywords Asthma • Mitochondria • Mitochondrial dysfunction • Apoptosis

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Introduction

Albert von Kölliker described some intracellular parts (now mitochondria) present in muscle cell as "granules" in 1857, then Richard Altman stained and named them as "bioblasts" in 1886, and finally Carl Benda coined them as "*mitochondria*" (mitos: thread, chondros: granule) [1]. Today we know that the humble mitochondrion, a primitive structure believed to have evolved from α-proteobacteria, is not merely a powerhouse of the cell but its function could regulate the function of whole organism [2]. This has led to its emergence as a key organelle in several chronic and metabolic diseases. Both basic and applied researchers are excited in exploring mitochondrial biology, thanks to the recognition of novel functions and features of mitochondria in addition to energy synthesis such as apoptosis, oxidative stress, calcium regulation, and mitochondrial DNA mutation-mediated diseases.

The mitochondrion has solute permeable outer membrane, intermembranous space, solute impermeable inner membrane, and matrix. The inner mitochondrial membrane is embedded with respiratory chain enzymes that utilize electrons from NADH or FADH2 as energy to create a proton gradient in the intermembranous space, which further drives ATP production by ATP synthase [2]. While the mitochondria have a separate genome that replicates independently, mitochondrial DNA can synthesize only 13 proteins for electron transport chain, whereas proper mitochondrial structure and function need more than a thousand proteins; so mitochondria have sophisticated protein import machineries on both membranes to import nucleus-encoded mitochondrial precursor proteins with mitochondrial targeting signals from cytosol [3]. In addition to the nucleus, the mitochondrion has a tight functional connection with most of the compartments of the cell and physical contact with few crucial organelles such as the endoplasmic reticulum, indicating that mitochondrion could be the point of convergence for cellular stress signaling [4–6].

Asthma (is a Greek word meaning "panting") affects a significant portion of the population worldwide and represents a significant health and economic burden [7, 8]. Asthma is a syndrome rather than a single disease as it has various phenotypes; however, most of these phenotypic features converge in episodic airway obstruction and hyperresponsiveness [9]. Asthma is symptomatically characterized by difficulty in breathing, wheezing, coughing, increased sputum production, and chest pain. It is pathophysiologically characterized by airway hyperresponsiveness, airway inflammation including airway eosinophilia, increased IgE, mucus hypersecretion, and structural changes of the airway called airway remodeling [10]. Current definition of asthma is based on three components: chronic airway inflammation, reversible airflow obstruction, and enhanced bronchial reactivity [10]. Asthma symptoms are commonly triggered by airway irritants such as air pollution and smoke, allergens, respiratory infections, certain drugs, exercise, etc. Although the majority of asthmatics can be managed with available anti-asthma medications, complex phenotypes such as obese asthma, neutrophilic asthma, and steroid-resistant asthma are poorly understood and challenging to manage effectively [11–14]. Thus, there is a need to identify alternate pathogenetic pathways to unravel the complexity and devise new therapies.

Asthma is not considered to be a mitochondrial disease or syndrome. Most mitochondrial syndromes are characterized by neurological and/or muscular abnormalities, since the high metabolism of neurons and muscle cells requires a large functional mitochondrial mass, without which they would lose function. While neurons and smooth muscle cells are implicated in asthma pathogenesis (neurohumoral control, bronchial smooth muscle, and cellular dysfunction with increased release of mediators), the observed defects are of excessive function, e.g., excessive vagal tone or smooth muscle contraction [15, 16]. While other commonalities between asthma pathogenesis and mitochondrial biology exist, namely, (a) maternal transmission in some studies, (b) oxidative/nitrative stress and antioxidant defense, (c) apoptosis/necrosis, and (d) calcium ion homeostasis, these are largely nonspecific. Reliable associations between mitochondrial dysfunction and asthma came from reports of abnormal mitochondria in the bronchial epithelia of asthmatic children and mice [17, 18]. The bronchial epithelium is a primary site of injury in asthma, and epithelial cell apoptosis is a critical event in asthma pathogenesis. Since apoptosis signaling is classically composed of two pathways, a direct pathway from death receptor ligation and an alternative pathway mediated by mitochondria during cell stress, mitochondrial abnormality could precipitate into the development of asthma. This was experimentally shown in mice, when mice with preexisting mitochondrial dysfunction were found to be more susceptible to allergic airway inflammation and developed more severe disease [19, 20].

The findings discussed above provided an association between mitochondrial dysfunction and asthma as well as a possible mechanism, opening the possibility that restoration of mitochondrial health may be beneficial in asthma. It had previously been shown that a mitochondria-targeted antioxidant, coenzyme Q10, reduced corticosteroid requirement in asthmatic patients [21]. The benefits of targeting mitochondria with antioxidants such as vitamin E and N-acetyl-L-cysteine (NAC) in allergic diseases [22, 23] supported the possible involvement of mitochondria in allergic diathesis. In this chapter, we discuss comprehensively the role of mitochondria in asthma pathogenesis based on the findings from our lab and others.

Mitochondrial Genetics and Asthma

Every mitochondrion contains 2–10 mtDNA copies, and in humans each copy has approximately 16,569 nucleotides, which can generate only 37 genes in its own: 22 tRNAs, 13 polypeptides which are important in electron transport chain, and two rRNAs (12S and 16S rRNA) (Fig. 3.1). Mitochondrial DNA has a higher tendency for mutations as the mitochondrion does not have efficient DNA repair mechanisms and the mitochondrion itself is the major source of oxidative free radicals [24]. As mitochondria from paternal sperm are not contributing in the generation of fertilized ovum, at least in mammals, maternal inheritance is the common feature in many mitochondrial DNA mutation-mediated syndromes [25]. From a genetic standpoint, asthma is a complex disorder with more than a hundred genes

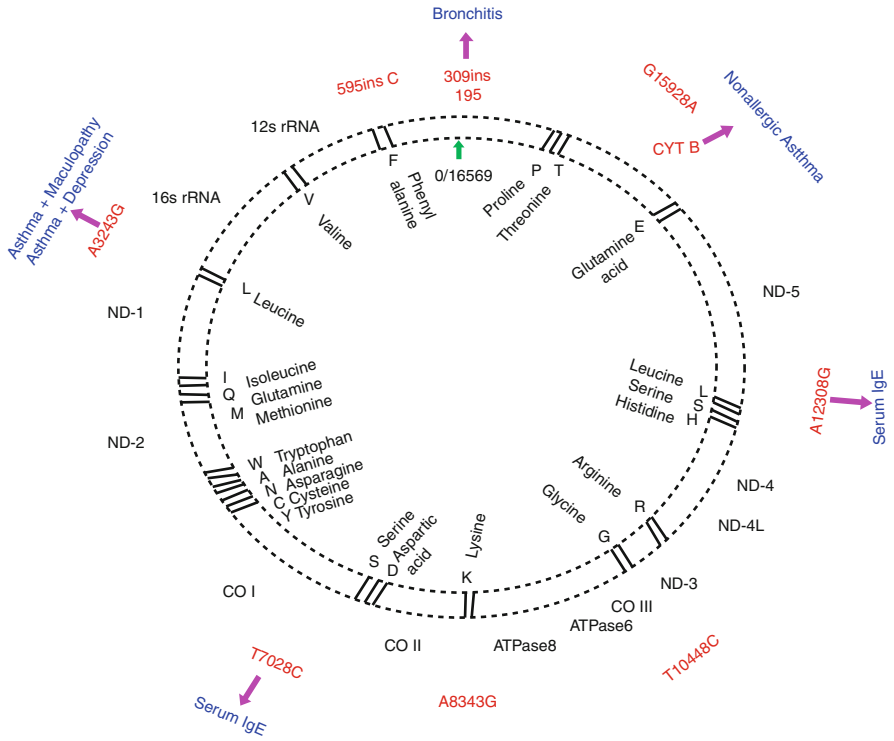

Fig. 3.1 Schematic diagram of human mitochondrial genome to show the mtDNA-derived 13 polypeptides, 22 tRNAs (each was named based on respective amino acid), and two rRNAs (12S and 16S). It also shows the reported mutations/polymorphisms in asthmatics along with respective associated asthma phenotypes

implicated so far. While asthma runs in families, it does not always follow Mendelian patterns, and different genes may be relevant in different individuals [26–28]. Though asthma is not considered as a mitochondrial disease, maternal history of asthma is one of the substantial risk factors for the development of asthma [29, 30] compared to paternal. Similar maternal influence has been observed in other atopic diseases such as atopic dermatitis, allergic rhinitis, etc. [31, 32]. Since maternal transmission is the hallmark of mitochondrial diseases, mitochondria could be important in the inheritance of asthma or atopy risk. Interestingly, maternal transmission of asthma features, such as airway hyperresponsiveness and increased susceptibility to allergic airway inflammation, was noted in mice. Interleukin-4 (IL-4), one of the Th2 cytokines crucial for most of the features of asthma, was shown to be involved in this transmission [33]. We have shown that administration of IL-4 monoclonal antibody reduces mitochondrial dysfunction and ultrastructural changes of bronchial epithelial mitochondria, suggesting that IL-4 may influence mitochondrial function [19]. IL-4 is also involved in IgE class switching, and importantly maternal IgE levels are well correlated with child cord blood IgE levels [34].

However, while this supports a possible role for mitochondria, various environmental factors such as transplacental passage of molecules and epigenetic factors are also likely to be involved in maternal mode of inheritance [35]. Interestingly, recent microarray studies performed in the placenta have revealed that various crucial mitochondrial genes are altered in the existence of maternal asthma during pregnancy, and glucocorticoid usage for asthma treatment during pregnancy also altered mitochondrial genes [36].

In support of a direct role for mitochondria in asthma, common mitochondrial haplogroups, especially haplogroup U from European population, have shown association with increased serum IgE levels [35]. Another study found five mutations in mitochondrial gene-encoding tRNAs (A12308G in tRNA leucine (CUN), G15928A in tRNA threonine, 595insC in tRNA phenylalanine, T10448C in tRNA arginine, and A8343G in tRNA lysine) and five more mutations in tRNA flanking regions [37]. Importantly, A12308G mutation is part of the mitochondrial haplogroup U, which was found to be significantly associated with total serum IgE levels. Another case-control study in Caucasian European children has found an association of the genomic region containing the ATPAF1 (ATP synthase mitochondrial F1 complex assembly factor 1) gene in Caucasian European children [38]. Further, they found that ATPAF1 gene expression was increased in asthmatic bronchial biopsies [38]. In other studies, peripheral blood mononuclear cells (PBMCs) of allergic patients were found to differentially express nine mitochondrial genes, including II and III subunits of cytochrome c oxidase [39]; cytochrome b gene polymorphism was linked with nonallergic bronchial asthma [40]; and substitution polymorphism at 195 site and insertion polymorphism at 309 site of hypervariable regions of D loop of mitochondria were associated with bronchitis in children [41]. Interestingly, A3243G tRNALeu(UUR) mutation which is associated with MIDD (maternally inherited diabetes and deafness syndrome) and MERRF (myoclonic epilepsy and ragged-red fibers) syndromes has been found in rare forms of asthma along with other comorbidity conditions such as maculopathy or depression [42, 43]. It is to be noted that A3243G tRNALeu(UUR) leads to severe combined respiratory chain assembly defect with abnormal incorporation of amino acids, alteration in stabilization, methylation, aminoacylation, and codon recognition of tRNA [44]. These indicate the necessity of initiating a study of mitochondrial variome in various populations to understand the exact role of mitochondria in the pathogenesis of asthma and other diseases.

Mitochondrial Function and Asthma

It is also necessary to note that mitochondrial research has seen periodic surges related to discoveries of new functions of mitochondria, starting from initial descriptions as energy sources to critical orchestrators of cell death [45]. Meanwhile, the understanding and management of asthma have also seen various evolutions (from the concept of smooth muscle defect to airway remodeling), thanks to the rapid

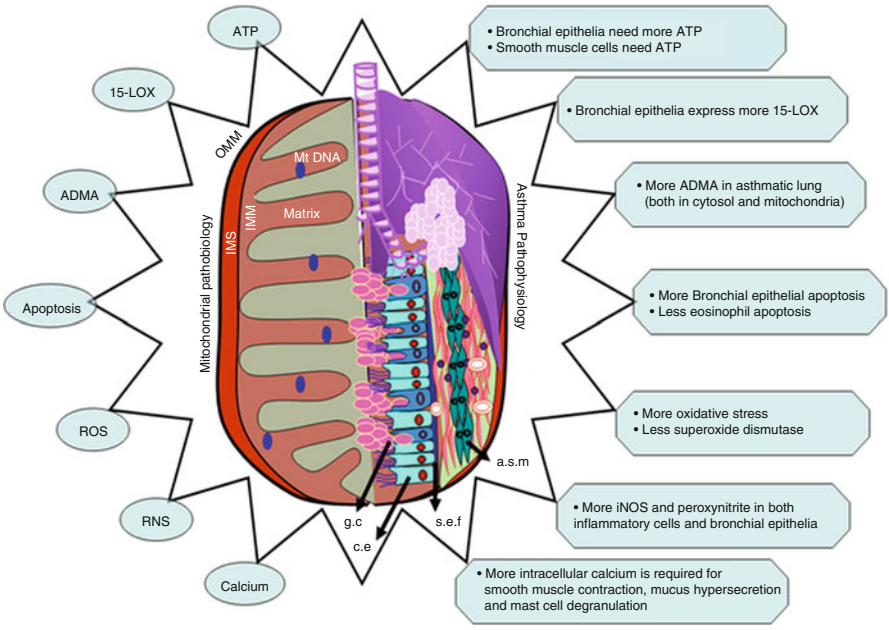

Fig. 3.2 Schematic diagram shows the respiratory organelle of the cell on one side and respiratory organ of body on the other side to indicate the possible common aspects between mitochondrial biology and asthma pathogenesis. These are ATP generation, 15-lipoxygensase (*15-LOX*), asymmetric dimethyl L-arginine (*ADMA*), apoptosis, reactive oxygen free radicals (*ROS*), reactive nitrogen free radicals (*RNS*), and calcium homeostasis. *OMM* outer mitochondrial membrane, *IMS* intermembranous space, *IMM* inner mitochondrial membrane, *g.c*. goblet cell, *c.e*. ciliated epithelia, *s.e.f*. subepithelial fibrosis, *a.s.m*. airway smooth muscle

advances in the field of immunology and molecular biology [46, 47]. While a link between the two was not realized until recently, many common features can be seen between mitochondrial pathobiology and asthma pathophysiology (Fig. 3.2).

Mitochondrial Dysfunction

Before our lab demonstrated the potential role of mitochondrial dysfunction in asthma, there were scattered reports indicating increased number of mitochondria in the bronchial epithelia of asthmatic children and experimental allergic mice [17, 18]. The functional status of such mitochondria was not known. We found that allergic murine lungs show features of mitochondrial dysfunction like decreased cytochrome *c* oxidase activity in lung mitochondria, decrease in the expressions of the third subunit of cytochrome *c* oxidase and 17 kDa subunit of complex I in bronchial epithelium, decreased lung ATP levels, and increased cytochrome c in lung cytosol [19].

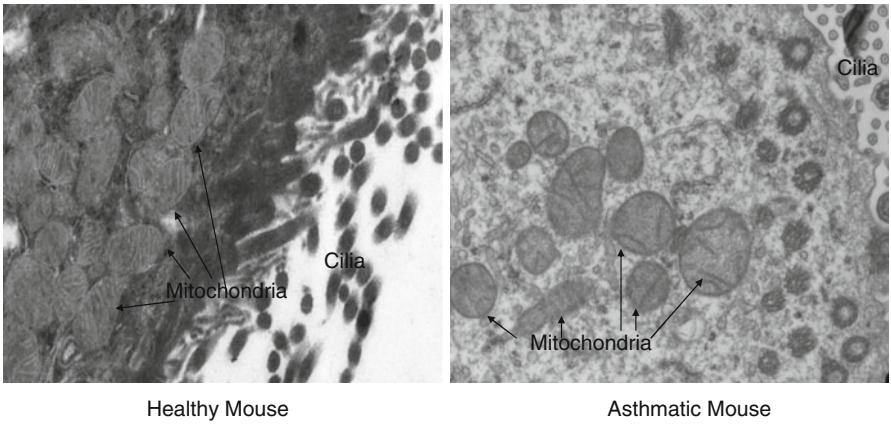

Fig. 3.3 Transmission electron microscopy of bronchial epithelia of asthmatic mice

Importantly, this was associated with mitochondrial ultrastructural changes such as mitochondrial swelling and loss of cristae. Further, IL-4 monoclonal antibody administration reduced these mitochondrial dysfunction and ultrastructural changes, indicating that mitochondrial dysfunction is associated with asthma pathogenesis (Fig. 3.3) [19]. In another study, mice exposed to environmental irritants such as tobacco smoke and printer emissions exhibited damage to lung mitochondria along with increased generation of reactive oxygen species (ROS) [48]. However, whether the observed mitochondrial dysfunction was the cause or effect in asthma pathogenesis remained uncertain. Findings from Boldogh lab shed some light in this context, where they demonstrated that increase in ROS generation from mitochondrial respiratory complex III augments Th2 responses independent of the adaptive immune response [49]. Further, they demonstrated that preexisting mitochondrial dysfunction in airway epithelia which was induced by deficiency of UQCRC2, ubiquinol-cytochrome c reductase core II protein, worsened asthma features in ragweed pollen-induced allergic mice [20]. These data indicate that mitochondrial dysfunction may be an important component of asthma pathogenesis and that restoration of normal mitochondrial function may be an important target for improving asthma. In support of these findings from mouse studies, dysfunctional mitochondria have also been observed in human asthmatic bronchial epithelia [50].

Reactive Oxygen Species

It is a well-known fact that mitochondria-mediated aerobic metabolism is, bioenergetically, at least 19 times more efficient than anaerobic glycolysis [51]. However, this energetically favorable system also introduces oxidative free radicals as

unwanted by-products. Mitochondria are the major endogenous source of oxidative free radicals, and oxidative stress is tightly linked with airway inflammation. Additionally, asthmatic airway is bombarded with various proinflammatory mediators from various recruited inflammatory cells such as eosinophil, lymphocytes, macrophages, neutrophil, mast cells, etc. This leads to increased oxidative stress in the lung. For example, eosinophil secretes various toxic proteins such as eosinophil peroxidase, major basic protein, eosinophil cationic protein, and eosinophil-derived neurotoxin [52]. These various proinflammatory mediators create local oxidative milieu in the airway [53]. The fate of generated superoxide (O_2^-) or free radicals is largely dependent on the local microenvironment. If there are sufficient dismutase enzymes inside the mitochondria, superoxide (O_2^-) is converted to H_2O_2, which is relatively stable and can be further broken down by catalase. However, superoxide dismutase has been shown to be reduced in asthmatic airway [54, 55]. Under these conditions, unstable superoxide may directly damage the mitochondria. This is consistent with the reports of mitochondrial dysfunction in asthma [19, 20].

The molecular targets of mitochondria-generated superoxide (O_2^-) are similar to any other parts of the cell such as nucleic acids, proteins, and lipids, similar to the NADPH oxidase-derived ROS. However, since mitochondria lack sophisticated DNA repair mechanisms, mitochondrial DNA mutations can accumulate [56]. Mitochondrial damage due to accumulated mitochondrial DNA mutations is an attractive theory of aging [57]. However, whether the reduction in the mitochondrial function due to accumulated mutations leads to aggravation of existing asthma features or induction of new asthma is yet to be investigated. To support this, various studies have found association of mitochondrial gene variations with asthma [35–42].

Oxidative damage to mitochondrial lipids such as cardiolipin, which is vulnerable due to its unsaturated state, leads to inhibition of the respiratory complexes [58, 59]. Oxidative stress also induces prooxidant enzymes such as 15-lipoxygenase (15-LOX), a lipid-peroxidizing enzyme, which is present in cytosol [60]. 15-LOX is unique since it directly hydrolyzes phospholipid esters present in biomembranes such as mitochondrial membrane even without the prior action of phospholipase A_2 (PLA_2) [60]. A number of evidences show that 15-LOX may be the common denominator between asthma pathogenesis and mitochondrial dysfunction. 15-LOX is the main enzyme in degrading mitochondria in reticulocyte during RBC maturation, attacks mitochondria under oxidative stress conditions, and can lead to mitochondria-mediated apoptosis [59]. In the lungs, 15-LOX is inducible by important Th2 cytokines such as IL-4 and IL-13, which are sufficient to induce an asthma-like state. Further, 15-LOX is predominantly expressed in the bronchial epithelium, where mitochondrial dysfunction and apoptosis have been noted [60]. In mice, deficiency of 12/15-LOX has been shown to alleviate the features of asthma [61, 62]. Together, this suggests that IL-4- or IL-13-mediated increase in 15-LOX may cause epithelial mitochondrial dysfunction and injury, which in turn lead to asthma. However, neither the exact role of 15-LOX in mitochondrial degeneration nor the consequences of epithelial mitochondrial dysfunction that leads towards asthma pathogenesis are well understood.

Reactive Nitrogen Species

Nitric oxide synthase (NOS) generates NO from substrate L-arginine, a semi-essential amino acid, and there are different isoforms of NOS: constitutive and inducible [63]. The role of NO in asthma is controversial and depends on its cellular source, isoform, and microenvironment [63, 64]. In normal airways, NO from constitutively expressed NOS such as eNOS (endothelial NOS) reduces airway tone by bronchodilation via cGMP pathway and increases mitochondrial biogenesis [63, 64]. In asthmatic airways, high levels of arginase consume most of the endogenous L-arginine and thus lead to reduced substrate availability to eNOS, inhibiting its bronchodilator action. Increased expression of iNOS during inflammation along with high levels of ROS leads to formation of reactive nitrogen species, peroxynitrite, which can cause numerous cytotoxic activities including mitochondrial dysfunction and is also a potent bronchoconstrictor [63]. NO and peroxynitrite inhibit cytochrome c oxidase (COX), a key oxidative enzyme of mitochondrial electron transport chain [65, 66]. Peroxynitrite not only affects cytochrome c oxidase but also affects many other mitochondrial enzymes such as NADH/coenzyme-Q reductase, aconitase, cytochrome *c* reductase, ATP synthase, and succinate dehydrogenase [67]. Thus, peroxynitrite is more effective to cause mitochondrial damage compared to NO. Thus, NO has a dual role in asthma pathophysiology as well as mitochondrial biology. In this context, therapeutic strategies for increasing eNOS and/or decreasing iNOS and peroxynitrite would be beneficial for both asthma and mitochondria. In this context, a high dose of L-arginine (250 mg/kg) reduces the formation of peroxynitrite by regulating NO and asymmetric dimethylarginine (ADMA) metabolism and alleviates the asthma features along with the reduction in mitochondrial dysfunction and epithelial damage [64, 68–70]. Since the existence of mitochondrial NOS is still under debate, we are not discussing that here [71].

Increased peroxynitrite formation in asthma can also be explained by the high levels of asymmetric dimethyl arginine (ADMA). ADMA binds to NOS, displacing L-arginine, and cannot be oxidized to NO, instead leading to formation of superoxide by uncoupling electron flow and NO production [69]. Although it has been suggested that ADMA can inhibit all forms of NOS, it predominantly inhibits eNOS in airway epithelia compared to inducible NOS [63, 68, 69]. The uncoupling of NOS leads to production of superoxide and peroxynitrite, which leads to mitochondrial dysfunction in pulmonary arterial endothelial cells [72]; however, the exact role of ADMA in asthma is yet to be explored. We have shown increased ADMA levels, along with increased expression of ADMA-synthesizing enzyme and decrease in the expression of ADMA-degrading enzyme, in bronchial epithelia of asthmatic lungs [69]. Importantly, ADMA levels were found to be increased in lung mitochondria in asthmatic mice [69]. Also, ADMA infusion leads to increased collagen deposition and deterioration of lung function in mice [73]. Further, ADMA increases the levels of both superoxide free radicals and peroxynitrite in bronchial epithelia (Fig. 3.4) [74].

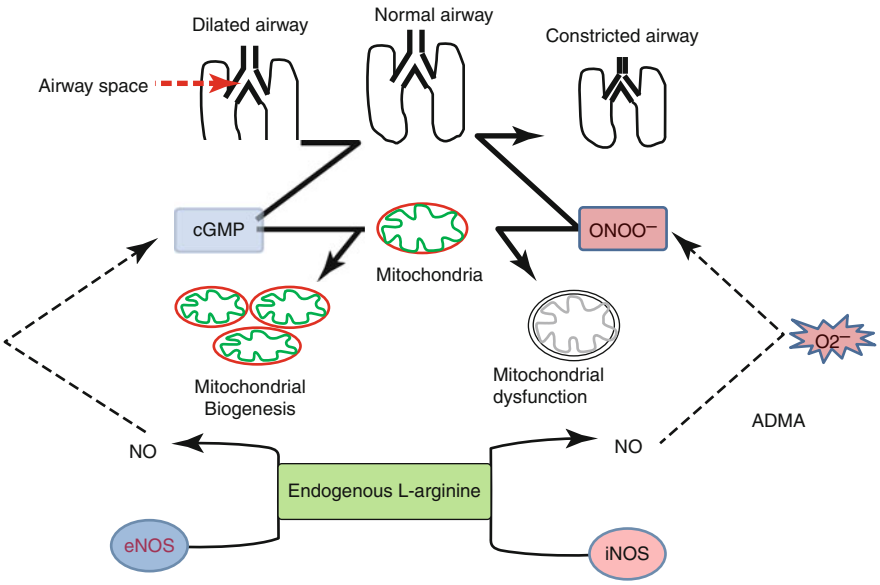

Fig. 3.4 Schematic diagram to show the dual role of nitric oxide (*NO*) in bronchial asthma and mitochondrial biology. Endogenous L-arginine is the common substrate for both eNOS (*endothelial NOS*) and iNOS (*inducible NOS*). NO derived from eNOS activates cGMP which dilate the airway; on the other hand cGMP also leads to mitochondrial biogenesis, whereas NO derived from iNOS reacts with superoxide free radicals to form peroxynitrite ($ONOO^-$) which is the powerful bronchoconstrictor and also causes mitochondrial dysfunction. Increased ADMA leads to increase in the formation of superoxide free radicals and peroxynitrite

Apoptosis

Epithelial injury and shedding are increased in asthma. This may relate to increased mitochondria-mediated apoptosis in bronchial epithelia as oxidative stress in the lung could lead to cardiolipin peroxidation that dissociate cytochrome c from cardiolipin and release into cytosol where cytochrome c complexes with Apaf-1 and deoxy-ATP to form apoptosome which further activates caspase 9 and caspase 3 to execute apoptosis [75, 76]. In this context, increase of both caspase 9 and DNA fragmentation in asthmatic patients and OVA-induced mice were observed [77]. Further, the disturbance in the balance between apoptotic and antiapoptotic members of Bcl2 has been demonstrated in asthma [78]. It has been shown that exposure of bronchial epithelial cells to TNF-alpha and IFN-gamma leads to the damage of mitochondria and consequently epithelial injury [79]. Supportively, mucous cell apoptosis has been induced by IFN-gamma by BAX upregulation and its translocation into mitochondria [80], indicating that mitochondria-mediated apoptosis could be independent of Th1/Th2 types of inflammation. However, mitochondrial-independent pathways are also likely to be important in epithelial apoptosis. The mitochondrial-apoptotic pathway is also crucial for programmed death of

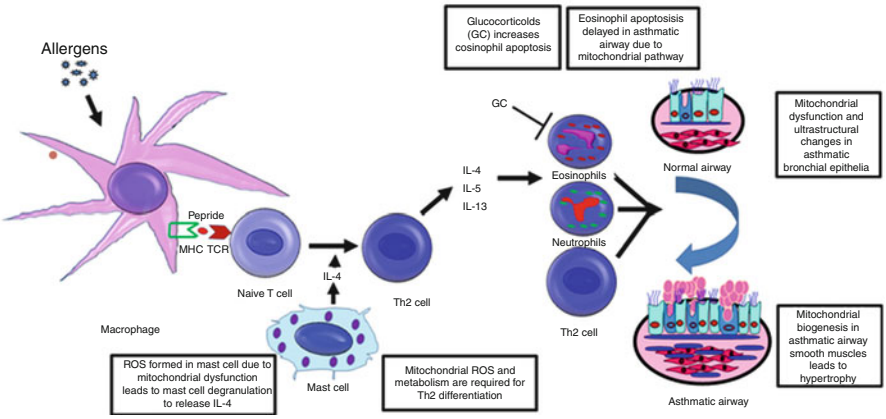

Fig. 3.5 Schematic diagram to show the involvement of mitochondria at various stages of asthma pathophysiology. Mitochondrial dysfunction occurs in mast cells leading to generate ROS which degranulates mast cell to release IL-4 that converts naïve T cells to Th2 cells. These Th2 cells release various cytokines and recruit various inflammatory cells such as eosinophils, neutrophils, and lymphocytes. Recruited inflammatory cells release several proinflammatory mediators which injure airway epithelia. Epithelial injury activates epithelial mesenchymal trophic unit (EMTU) to initiate airway remodeling which consists of hyperplasia and hypertrophy of smooth muscle, goblet cell metaplasia, and subepithelial fibrosis. Asthmatic bronchial epithelia had shown the features of mitochondrial dysfunction, whereas asthmatic airway smooth muscle had shown the features of mitochondrial biogenesis

immune cells during resolution of asthma [81, 82]. Interestingly, glucocorticoids have been shown to have proapoptotic effect on human eosinophils via the mitochondrial pathway [83]. Translocation of Bax to the mitochondria, release of cytochrome c, caspase-independent collapse of the mitochondrial membrane, and subsequent activation of caspases are involved in spontaneous eosinophil apoptosis [84]. Failure of inducing eosinophil apoptosis by steroid could be one of the mechanisms of steroid resistance in severe asthmatic patients [85, 86]. This subgroup of asthmatic patients is difficult to treat and consumes a disproportionately large share of health resources [87]. Thus, investigating the role of mitochondria in steroid resistance is an important area of research (Fig. 3.5).

Airway Remodeling

The important sequelae of bronchial epithelial injury is airway remodeling [88–90]. Repeated allergen exposures lead to structural alterations in the airway, called airway remodeling. Airway epithelia have been postulated to contribute to a large extent to the remodeling process [91]. The airway epithelium in asthmatics is injured by inhaled pollutants, allergens, viruses, and also by endogenous factors such as proteolytic enzymes [89]. Injured epithelium activates epithelial mesenchymal trophic unit (EMTU) [90]. EMTU is active in the lung development at the embryonic

stage and it maintains the relationship between epithelia and mesenchymal cells with various growth factors such as TGF-beta, FGF, VEGF, etc. EMTU remains inactive in the adult lung and reactivated by epithelial injury, and its activation in asthmatic airway releases various growth factors which are responsible for various components of airway remodeling. Airway remodeling, a complex phenomenon, consists of alteration in the number and/or type of resident cells and the proportion/quantitation of various components of extracellular matrix [90]. Airway remodeling includes epithelial damage, epithelial hypertrophy and hyperplasia, goblet cell metaplasia, airway myofibroblast transformation, increased airway collagen deposition, hyperplasia, and hypertrophy of airway smooth muscle and bronchial blood vessels [91, 92]. Among these features, hyperplasia and hypertrophy of airway smooth muscle and subepithelial fibrosis are crucial components. On the one hand, there is more of proline causing subepithelial fibrosis [93], and on the other hand, proline leads to inhibit cytochrome c oxidase [94]. Bronchial smooth muscle of asthmatics showed more number of mitochondria with increase in the factors required for mitochondrial biogenesis such as peroxisome proliferator-activated receptor-γ coactivator, nuclear respiratory factor 1, and mitochondrial transcription factor [95].

It was further demonstrated that mitochondria are responsible for smooth muscle proliferation which emphasizes the importance of mitochondria in airway remodeling [95]. Also, mitochondrial biogenesis and increased mitochondrial activity have been reported in asthmatic airway smooth muscle along with altered calcium homeostasis, and it has been suggested that these are crucial in smooth muscle hypertrophy of airway remodeling [95]. However, mitochondrial dysfunction was not observed in those mitochondria from bronchial smooth muscle [95]. In contrast, our lab and others found mitochondrial dysfunction in bronchial epithelia [19, 20]. These indicate the possibility of differential roles of mitochondria in the lung. Thus, it seems there is a differential status of mitochondria in different cell types of asthmatic lungs, and thus careful interpretations are required to understand the precise role of mitochondria in asthma pathogenesis. This is very crucial in converting this knowledge into therapeutics. For example, theophylline causes cAMP-dependent tyrosine phosphorylation of subunit I of cytochrome c oxidase (COX) leading to its inhibition in smooth muscle cells [96]. If this is not cell-specific, it also could inhibit COX in bronchial epithelia and might create havoc in epithelial homeostasis. Thus, meticulous investigations are needed to take mitochondrial research in asthma from bench to the bedside in the form of mitochondrial medicine. It also has been suggested that both mitochondrial dysfunction and mitochondrial biogenesis are interrelated with each other as the latter feature is the possible compensatory mechanism of the former. In addition, it has been suggested that the dense localization of benzodiazepine receptor (BR) on the outer mitochondrial membrane of airway smooth muscle might have a role in asthmatic AHR [97]. Interestingly, airway smooth muscle is known to have high density of mitochondrial benzodiazepine receptor (MBR), a peripheral benzodiazepine receptor (PBR). There are two major types of BR, CBR (central type) and PBR. CBR is present exclusively in neurons, located at the plasma membrane, and regulates chloride channel intrinsic to the $GABA_A$ receptor, which is responsible for the antianxiety, anticonvulsant, and muscle relaxant effects of benzodiazepines [97].

PBR is distributed ubiquitously and predominantly located at the outer mitochondrial membrane; due to this reason PBR is generally called as MBR. The exact physiological role of PBR is not clear but has been postulated to relate to mitochondrial heme synthesis and steroid synthesis. Indeed, specific MBR ligands have been shown to affect the airway smooth muscle contraction in basal as well as pharmacologically stimulated conditions [97]. Also, these MBRs are densely localized in various structures in the lung other than airway smooth muscle, such as airway epithelium and submucosal glands. Further studies are needed to better define the potential role of MBR in the modulation of bronchial caliber and in the pathogenesis of asthma.

Calcium Homeostasis and Asthma

It is well known that calcium plays an integrated role in asthma pathogenesis as most of the pathophysiological features such as airway smooth muscle contraction, mast cell degranulation, mucus secretion, recruitment of inflammatory cells, release of proinflammatory mediators from inflammatory cells, and vagal neurotransmission are dependent on calcium signaling. Indeed, calcium channel blockers have been used in asthmatic patients where beta adrenergic receptor agonists were contraindicated or ineffective [98]. Calcium signals also influence most of the functions of mitochondria such as oxidative phosphorylation, apoptosis, etc. [99]. Other than signaling, the mitochondrion acts as an intelligent calcium buffering system since they can sense and fine-tune intracellular calcium levels. Increased intracellular calcium leads to enhanced mitochondrial uptake of calcium via uniporters present in the inner mitochondrial membrane, and this uptake further regulates the calcium release from the plasma membrane and endoplasmic reticulum. Thus, mitochondria can act as buffering organelles in a spatiotemporal manner to maintain the calcium homeostasis inside the cell [100]. This mitochondrial calcium uptake leads to reduction of intracellular calcium, preventing excitotoxicity in cells. However, mitochondrial calcium overload itself may lead to mitochondrial damage and possibly cell death if there is significant damage caused to many mitochondria in a cell [101, 102]. Though calcium signaling is important for both mitochondrial physiology and asthma pathogenesis, detailed studies of this aspect are still lacking. It is to be noted that *ORMDL3*, one of the very few genes identified through genome-wide association study in asthma, binds and inhibits sarco-endoplasmic reticulum Ca^{2+} (SERCA) pump and alters endoplasmic reticulum-mediated calcium homeostasis [103].

Viral Infections and Asthma

Respiratory viral infection is one of the risk factors for asthma development in children [104]. Concurrent viral infections in asthma aggravate asthma features, cause acute exacerbations in chronic asthmatic conditions, and also lead to irreversible

stage of airway remodeling [105]. Though it is very clear that viral infections caused by respiratory syncytial viruses, and few other viruses of the paramyxoviridae family, worsen airway inflammation and airway hyperresponsiveness, the mechanisms underlying this remain poorly understood. The bronchial epithelium is the major site of viral infections in the airway and participates in innate immune responses to viral infections [106]. Interestingly, mitochondria are suggested as surveillance sites for viruses due to the presence of antiviral proteins in mitochondria [107–109] such as MAVS (named for its *m*itochondrial *a*nti*v*iral *s*ignaling). Nucleic acids of various paramyxoviruses are recognized intracellularly by cytoplasmic receptors such as by RIG-I (retinoic acid-inducible gene I)/melanoma differentiation-associated protein 5 (MDA5) which further binds with MAVS present in outer mitochondrial membrane through CARD-CARD (caspase activation and recruitment *domains*) interactions [110, 111]. MAVS further recruits other molecules such as Tom70, and TNF-receptor-associated factors to assemble signalosome complex and activate IKK and TBK-1 complexes to signal both NF-κB and IRF3 signaling pathways to further initiate type I interferon responses to evade viral pathogens [110, 111]. In this signaling, mitochondrial localization of MAVS is very crucial as removal of MAVS from mitochondria evades the host immune system. Whether viral infection-induced mitochondrial stress triggers asthma related pathways, or mitochondrial dysfunction in asthmatic airway epithelium accentuates viral infection, remains to be studied.

Therapeutics

The studies discussed above indicate strongly that mitochondrial dysfunction is an important aspect of asthma pathophysiology. It is therefore important to evolve strategies for mitochondrial protection as part of efforts to promote lung health and alleviate asthma. At this time, there are many gaps in the available knowledge, and further investigations are required to develop a sufficient understanding of the interface between mitochondrial biology and lung disease. Numerous efforts are being taken to bring mitochondrial bench research to bedside to control/cure various diseases [112–114]. As a result "mitochondrial medicine" was introduced with the recognition of new target sites in mitochondria. There are two major types of drug targets in mitochondria: direct or primary which acts specifically on mitochondrial enzymes and metabolic pathways and indirect or secondary which acts on mitochondria in nonspecific or accidental manner [115]. Possible reasons for secondary mechanisms are structural homology between mitochondria and other cellular parts, bacterial origin of mitochondria, functional or structural cross talk between mitochondria with other cellular compartments, etc. For example, the secondary effects of various antibiotics like chloramphenicol which affect protein synthesis of both bacteria and mitochondria may be explained by the bacterial origin of mitochondria [116]. The capacity of stem cells in donating mitochondria in rescuing injured airway epithelia in acute lung injury condition indicates another emerging target through the healthy mitochondrial transfer to damaged cells [117]. Though various reports demonstrated

the therapeutic capacity of stem cells in asthma [118, 119], whether this is directly through the donation of mitochondria is yet to be demonstrated.

Our lab showed that mitochondrial dysfunction and mitochondrial structural changes, in asthma, were reversible with treatment of allergic mice with plant-derived coumarins (esculetin), vitamin E, or statins [120–122]. Esculetin treatment to experimentally allergic mice reduced asthma features such as airway hyperresponsiveness, airway inflammation, Th2 response, eotaxin, and airway eosinophilia along with restoration of mitochondrial function and structure in bronchial epithelia. It is important to note that both esculetin and vitamin E are not only antioxidants but they reduced the expression and activity of reticulocyte lipoxygenase in the lung. So we believe that esculetin might restore mitochondrial function through its primary cytosolic target, reticulocyte lipoxygenase. Coenzyme Q and vitamin E are mitochondria-targeted antioxidants. Notably, coenzyme Q10 is known to reduce the requirement of corticosteroids in asthmatic patients [21]. Further, using experimental mouse models, we have shown that L-arginine and statins alleviate asthma features and improve mitochondrial function in lungs, possibly due to restoration of homeostatic nitric oxide metabolism in bronchial epithelia [69, 122]. However, each of these observations is limited by inability to distinguish between primary and secondary effects. Thus, further efforts have to be taken to develop "mitochondrial medicine" for effective management of chronic respiratory diseases.

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Chapter 4
ROS Signaling in Cardiovascular Dysfunction Associated with Obstructive Sleep Apnea

Ganesh K. Kumar, Gayatri Raghuraman, and Nanduri R. Prabhakar

Abstract Obstructive sleep apnea (OSA) associated with recurrent apnea is one of the most commonly encountered breathing disorders in adult humans. Patients with OSA are prone to develop hypertension and cardiovascular diseases. Despite its adverse clinical consequences, the mechanism(s) by which recurrent apneas lead to cardiovascular abnormalities is poorly understood. OSA causes periodic decreases in arterial blood O_2 or intermittent hypoxia (IH). Available evidence suggests that exposing experimental animals to IH during sleep is sufficient to induce cardiovascular abnormalities similar to those seen in OSA patients. A majority of cross-sectional and prospective studies show that patients with severe OSA exhibit oxidative stress compared to healthy humans. Studies in experimental animals provide evidence for IH-induced ROS generation mediating cardiovascular dysfunction. IH-evoked ROS generation seems to involve activation of NADPH oxidase, inhibition of mitochondrial complex I, and downregulation of antioxidant enzymes. Recent studies have identified hypoxia-inducible factor-1 and 2 as major molecular determinants for sustained oxidative stress elicited by IH. Continuous positive airway pressure treatment, which reduces oxidative stress, appears to be effective in attenuating cardiovascular dysfunction in a *subset* of OSA patients.

Keywords Chronic intermittent hypoxia • NADPH oxidase • Mitochondrial complex I • Posttranslational modification • S-glutathionylation • Hypertension • Chemoreflex • Baroreflex • Sympathetic nerve activity • Oxidative stress • Prooxidant enzymes • Antioxidant enzymes • Hypoxia-inducible factor-1 • Hypoxia-inducible factor-2

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Abbreviations

AM	Adrenal medulla
CA	Catecholamines
CaMK	Calcium-calmodulin-dependent kinase
ERK	Extracellular signal-regulated kinase
ET-1	Endothelin 1
ETC	Electron transport chain
HIF-1α	Hypoxia-inducible factor-1α
HIF-2α	Hypoxia-inducible factor-2α
HVR	Hypoxic ventilatory response
IH	Intermittent hypoxia
MAP	Mean arterial pressure
MnTMPyP	Manganese(III) tetrakis(1-methyl-4-pyridyl)porphyrin
MSNA	Muscle sympathetic nerve activity
Nox	NADPH oxidase
$O_2^{\cdot-}$	Superoxide anion
OSA	Obstructive sleep apnea
PKA	Protein kinase A
PP2A	Protein phosphatase 2A
ROS	Reactive oxygen species
SNA	Sympathetic nerve activity
SOD	Superoxide dismutase
T-BARS	Thiobarbituric acid-reactive substances
TH	Tyrosine hydroxylase

Introduction

Obstructive sleep apnea (OSA) associated with recurrent apnea is a commonly encountered respiratory problem in adult humans and preterm infants. OSA affects an estimated 18 million people in the USA alone [27, 81, 84, 97, 112]. Patients with recurrent apneas exhibit autonomic abnormalities, including elevation in sympathetic nerve activity (SNA) which persisted in daytime wherein breathing is normal [49, 109, 110] and cardiovascular dysfunction including hypertension [18, 28, 73, 81, 84, 97, 112]. The mechanisms by which recurrent apneas lead to autonomic and cardiovascular dysfunction are not fully understood, and unraveling these mechanisms is a major focus of current research in the field of sleep-disordered breathing.

OSA associated with recurrent apnea results in intermittent hypoxia (IH), intermittent hypercapnia, and sleep fragmentation, which are important contributors to OSA-induced pathophysiology. In an earlier study, Fletcher et al. [36] reported that exposure of rodents to IH results in increased SNA and elevated mean arterial pressure (MAP). More importantly, addition of CO_2 during chronic IH had no significant augmentative effect on MAP [36]. Further, repetitive arousal of rodents from sleep without airway occlusion for 35 days had no significant effect on MAP [5].

These results suggest that hypoxia, rather than hypercapnia, is critical for inducing hypertension associated with recurrent apneas and that exposing experimental animals to IH is a valid model for examining the mechanisms that are activated by recurrent apneas associated with OSA.

Unlike IH, animals exposed to continuous hypoxia do not develop autonomic and cardiovascular abnormalities. During IH, each episode of hypoxia is followed by a reoxygenation phase which may give rise to generation of reactive oxygen species (ROS), similar to that reported during ischemia-reperfusion. Based on this similarity, Prabhakar [93] has proposed that IH induces ROS generation resulting in oxidative stress which in turn mediates autonomic and cardiovascular abnormalities. Since then, a large number of studies have examined the importance of ROS in autonomic and cardiovascular abnormalities seen in OSA patients and rodents exposed to IH.

In the following sections, we present an overview of studies examining (1) autonomic and cardiovascular functions in OSA patients and experimental animals exposed to IH, (2) evidence of oxidative stress, (3) sources and mechanisms of ROS generation, and (4) role of ROS in autonomic and cardiovascular abnormalities elicited by recurrent apneas.

Autonomic and Cardiovascular Functions in OSA Patients

Physiologically, heart rate and blood pressure are lower during sleep than during wakefulness because of vagal activation and vascular sympathetic withdrawal [12, 107, 111]. This sleep-dependent phenotype seems to be absent in OSA patients suggesting a possible association between OSA and arterial hypertension [79]. Cross-sectional studies suggest a potential link between OSA and arterial hypertension, a major risk factor for fatal and nonfatal cardiovascular events [13, 27, 48]. However, conflicting results were reported by two earlier prospective studies [82, 91]. The Wisconsin Sleep Cohort Study reported a correlation between the risk of incident hypertension and severity of OSA in middle-age patients [91]. Contrasting this finding, the Sleep Heart Health Study did not find a similar link in older individuals with severe OSA [82]. It is likely that these conflicting findings from these two studies may in part be due to differences in the follow-up period and the size of patients with severe OSA included in the study. However, in a recent study involving a median of 12.2 years of follow-up, Marin et al. [70] found a strong correlation between OSA and incident hypertension. It is likely that the assessment of causal link between OSA and hypertension is complicated by (1) shared risk factors (e.g., obesity which affects both OSA and hypertension), (2) variations in the susceptibility of individuals to the adverse effects of recurrent apneas, and (3) difficulties in the conduction of definitive randomized controlled trials [59]. Nonetheless, collectively the above results suggest an association between OSA and hypertension and further emphasize that such an association is difficult to detect in older adults.

There is also evidence that sleep apnea patients exhibit atherosclerosis [30] and endothelial dysfunction [55]. In addition to arterial hypertension, sleep apnea has

also been linked to development of ischemic heart disease, stroke [11, 32], arrhythmia, myocardial infarction [53], chronic heart failure, and other cardiovascular morbidity and mortality [42, 54, 80, 104]. Since elegant reviews addressing these cardiovascular abnormalities associated with sleep apnea are already available [4, 15, 42, 57, 91, 123], these aspects will not be elaborated further. In the following sections, the mechanisms underlying blood pressure elevation by IH associated with OSA will be discussed.

Mechanisms of Blood Pressure Regulation in OSA Patients

Imbalance in Chemoreflex-Baroreflex Function

Reflexes arising from the peripheral arterial chemo- and baroreceptors contribute to regulation of blood pressure by altering the sympathetic tone. Patients with OSA have elevated blood pressure and heightened sympathetic tone as measured by muscle sympathetic nerve activity (MSNA; [19, 49, 68, 110]) which persists even in daytime wherein breathing is normal. Arterial chemoreflex which is a major regulator of sympathetic nerve activity is augmented in patients with OSA as evidenced by (1) reduction of blood pressure by brief hyperoxic exposure, which inhibits chemoreceptor activity [78]; (2) augmented hypoxic ventilatory response, a hallmark chemoreflex response compared to controls [50]; and (3) greater activation of MSNA by apnea compared to control subjects [108].

Arterial baroreflex, on the other hand, exerts a tonic inhibitory influence on sympathetic activity. Available evidence suggests that baroreflex function is downregulated in patients with OSA as evidenced by attenuated heart rate and vascular response to baroreceptor activation [14, 26, 75].

Elevated Levels of Vasoconstrictors

In addition to chemo- and baroreflex regulation of blood pressure via sympathetic nervous system, several vasoactive hormones/mediators by directly acting on resistance vessels also alter blood pressure. In the periphery, the vascular endothelium, adrenal gland, and kidney produce the major vasoconstrictors, endothelins (from the endothelial cells), catecholamines (from chromaffin cells), and renin-angiotensin system (renal), respectively. Available evidence suggests that the levels of several vasoactive hormones/mediators are elevated either in plasma or in the urine of OSA patients compared to control subjects. Patients with OSA exhibit elevated plasma levels of norepinephrine and epinephrine [19, 38, 72], endothelin-1 [41, 92, 103, 127], and angiotensin II [39, 74] compared to healthy humans. However, few other studies reported no changes in plasma ET-1 levels in OSA patients [46, 74]. Taken together, the above studies suggest that OSA by profoundly altering the chemo- and baroreflex functions as well as elevating circulating levels of aminergic and peptidergic vasoconstrictors increases blood pressure (Fig. 4.1).

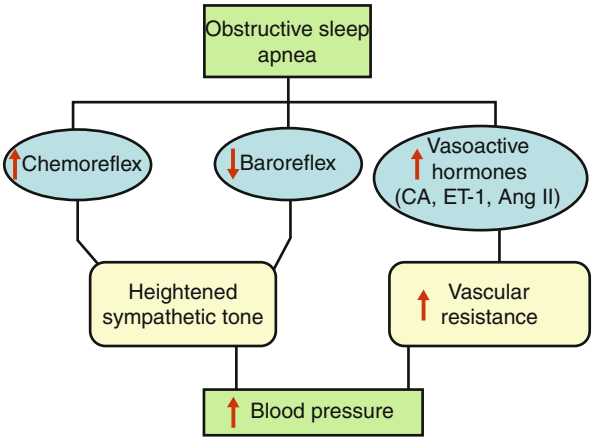

Fig. 4.1 Proposed mechanism of blood pressure elevation in patients with OSA

Effects of Continuous Positive Airway Pressure (CPAP) on Arterial Hypertension in OSA Patients

In OSA patients, nasal CPAP treatment eliminates repetitive episodes of hypoxia associated with transient cessation of breathing. Short-term nasal CPAP therapy induces a modest decrease in MAP in hypertensive OSA patients [13, 48], and a greater decrease in MAP is seen in patients with higher adherence to CPAP treatment, more severe OSA, and daytime sleepiness [70, 71]. In contrast, in patients with severe OSA without daytime sleepiness, CPAP treatment is found to be ineffective in lowering MAP [8] and reducing the incidence of hypertension or cardiovascular events [7]. Overall, these studies suggest that higher adherence to CPAP treatment is beneficial for patients with severe OSA exhibiting daytime sleepiness.

Autonomic and Cardiovascular Function in Rodents Exposed to IH

Advantages of Experimental Animal Models

Studies in experimental animals involving either rats or mice are very useful, and unlike human studies, they allow modulation of biological systems to elucidate detailed mechanisms of pathophysiology. This experimental approach offers several advantages. First, the effects of IH, a major contributing factor to OSA pathophysiology, can be investigated in the absence of any comorbidities or behavioral alterations normally seen in patients with OSA. Second, autonomic and cardiovascular variables as well as biochemical markers can be measured invasively under well-defined experimental conditions.

Table 4.1 Effects of different patterns of intermittent hypoxia on blood pressure (BP) in rats

Investigators	IH paradigm	Effects on BP	BP measurement via
Hui et al. [52]	10 and 21 % oxygen every 90 s during daylight hours	Increased BP after 30 days	Telemetry
Kanagy et al. [58]	N_2-CO_2 mixture for 90 s (5 % O_2–5 % CO_2) followed by 90 s of compressed air to achieve normoxia (21 % O_2–0 % CO_2)	Increased BP after 7 days	Arterial catheters
Silva and Schreihofer [106]	40 s at 6 % O_2 every 9 min, 8 h/ day	Increased BP after 2 weeks	Catheters placed into the femoral artery and vein
Kumar et al. [63]	15 s of 5 % inspired O_2 followed by 5 min of room air (normoxia), 9 episodes per h and 8 h per day for 10 days	Increased BP after 7 days	Tail cuff
Knight et al. [61]	10 and 21 % oxygen every 75 s, 80 cycles/day	Significant increase in BP after 7 days	Telemetry
Zoccal et al. [129]	5 min of 21 % oxygen and 4 min of pure nitrogen 8 h per day for 35 days	Increased BP after 35 days	Catheters placed into the abdominal aorta
Fletcher et al. [37]	3–5 % nadir ambient oxygen every 30 s, 7 h per day for up to 35 days	Increased BP after 35 days	Both tail cuff and femoral arterial catheters
Joyeux-Faure et al. [56]	40 s with 5 % O_2 and 21 % O_2 for 20 s; 8 h during daytime, for 35 days	Increased BP after 5 weeks	Femoral artery

Elevation of Blood Pressure in Rodents Exposed to Different IH Patterns

To understand the mechanisms underlying recurrent apnea-induced autonomic and cardiovascular abnormalities, experimental animals exposed to several days of IH have been developed. A survey of the literature showed that investigators in the field have used different patterns of IH. A few of them have been frequently used to examine the consequences and mechanisms of recurrent apneas on autonomic and cardiovascular functions, and these IH patterns are shown in Table 4.1. From a comparative analysis of these studies, the following generalizations can be made: (1) exposure to IH increases blood pressure in rats [37, 63] and mice [90], and (2) the magnitude of the increase in MAP is dependent on the duration of IH, severity of hypoxia, and the duration of hypoxic and normoxic cycles. Nonetheless, these studies suggest that IH in experimental animals induces hypertension. Interestingly, IH-induced increase in blood pressure is absent in carotid body transected rats [37] suggesting that intact peripheral chemoreceptors are necessary for the systemic blood pressure responses elicited by IH.

Effect of IH on Sympathetic Nerve Activity

To assess the role of altered sympathetic tone and levels of vasoconstrictors to IH-induced increase in blood pressure, several studies examine the effects of IH on sympathetic nerve activity and chemo- and baroreflex function and measure the levels of circulating vasoactive hormones in experimental animals. Rats exposed to different patterns of IH showed an increase in cervical [45], renal [51], splanchnic [29], thoracic [131], and lumbar [69] sympathetic nerve activity. In addition, studies by Silva and Schreihofer [106] showed that following IH, the sympathetic nervous system is highly sensitive to stimulation of sciatic nerve and the nasal mucosa. Thus, IH exposure activates sympathetic nervous system similar to that seen in OSA patients.

Effect of IH on Chemo- and Baroreflex Functions

IH has been shown to augment HVR, a hallmark response of arterial chemoreflex in cats [101] and mice [90]. Furthermore, sympathetic response to hypoxia is also exaggerated in rats treated with IH [16, 51, 69], and this effect is abolished by chronic bilateral sectioning of the carotid body [35, 95]. Thus, intact peripheral chemoreceptors are required for the sympathetic responses elicited by IH.

Unlike the arterial chemoreflex, IH-treated adult rats show diminished baroreflex control of heart rate [65] and splanchnic sympathetic nerve activity [87]; however, baroreflex control of cervical nerve activity is unaffected [45]. An increase in baroreflex function is seen in juvenile rats exposed to IH [130] suggesting that chronic IH-induced baroreflex responses are age-dependent.

Effect of IH on Circulating Levels of Vasoactive Hormones

Elevation in circulating catecholamines (both norepinephrine and epinephrine) is also observed in IH-exposed rats [6, 63] and mice [90]. In addition, plasma ET-1 levels are higher in rats treated with IH combined with hypercapnia than in control rats [58], and ET type A receptor antagonist normalized blood pressure in these rats [1].

Effect of IH on Catecholamine Secretion-Synthesis Coupling in the AM

In addition to the sympathetic nerve terminals at the vasculatures, the adrenal medulla (AM) is a major source of circulating catecholamines. To assess whether the AM contributes to IH-induced increase in circulating catecholamines, Fletcher et al. [37] examined the effects of adrenal demedullation on IH-induced raise in plasma catecholamines and blood pressure response. Adrenal demedullation not

only prevents the increase in plasma catecholamines but also the elevation in blood pressure in rats exposed to IH, suggesting that catecholamine secretion from the AM plays a critical role in eliciting cardiovascular changes [6].

Given the importance of AM-derived catecholamines in IH-evoked increase in blood pressure, Kumar et al. [63] investigated whether IH alters catecholamine secretion from the AM. Their results show that IH not only increase the content but also augment basal release of norepinephrine and epinephrine from the AM. More importantly, acute hypoxia evokes a robust increase in catecholamine secretion in the AM from rats conditioned with IH, and these responses are absent in control rats. The stimulatory effect of IH on catecholamine secretion is selective to hypoxic stimulus because hypercapnia is found to be ineffective. The hypoxic sensitivity of the AM elicited by IH is associated with downregulation of neurogenically mediated catecholamine secretion as evidenced by reduced responses to nicotine and 2-deoxyglucose [63, 114]. These results suggest that IH evokes functional remodeling of the AM.

The IH-induced increase in catecholamine content in the AM is in part due to increase in enzyme activity of tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis [62]. The increase in TH activity evoked by IH occurs via activation of posttranslational mechanisms involving serine phosphorylation. The activity of TH is physiologically regulated via phosphorylation of one or more of the four serine residues (Ser-8, 19, 31, and 40) located at the N-terminal regulatory domain of the enzyme [31]. IH increases serine phosphorylation of TH at residues 31 and 40, and these changes are associated with upregulation of protein kinase activities [protein kinase A (PKA), extracellular signal-regulated kinases (ERK), and calcium-calmodulin-dependent kinase (CaMK)II] and a simultaneous downregulation of protein phosphatase 2A (PP2A) activities [62, 99]. Thus, IH-induced tilting of the balance between protein kinases and protein phosphatases which leads to sustained phosphorylation and activation of TH resulting in increased catecholamine synthesis (Fig. 4.2) which is necessary to support the increased demand caused by elevated basal and hypoxia-evoked catecholamine secretion (i.e., secretion-synthesis coupling).

Role of Oxidative Stress in OSA-Induced Autonomic and Blood Pressure Changes

The results presented thus far show that adult rats and mice exposed to IH exhibit augmented chemoreflex and depressed baroreflex functions, elevated sympathetic activity, elevated circulating vasoactive hormones, and increased blood pressure similar to those exhibited by OSA patients. How does IH associated with recurrent apneas induce such autonomic and cardiovascular changes? The alternating hypoxia and reoxygenation cycle occurring during IH resembles hypoxia-reperfusion injury, which is shown to induce oxidative stress. Based on this similarity, it has been proposed that reactive oxygen species (ROS) generated during the reoxygenation phase and the ensuing oxidative stress mediate the physiological responses to IH [93].

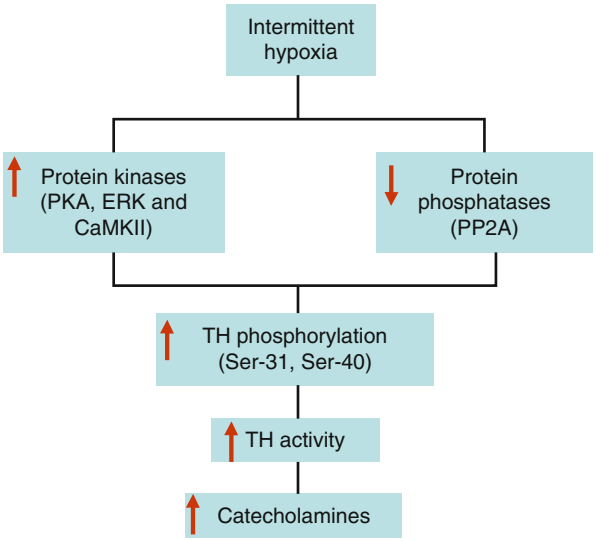

Fig. 4.2 Activation of posttranslational mechanisms in catecholamine synthesis during intermittent hypoxia

Oxidative Stress Markers

Various oxidative stress markers have been reported in the literature [116, 119]. ROS oxidize biological molecules forming lipid peroxides, protein carbonyls, and oxidized DNA products and also alter the levels of antioxidants including ascorbic acid, tocopherols, carotenoids, and flavonoids. Changes in the levels of one or more of these oxidation products and antioxidants have been used to assess the occurrence of oxidative stress. Alternatively, the degree of oxidation of biological samples by a known oxidant in vitro can also be used as a measure of oxidative stress. Using these approaches, several studies have examined the occurrence of oxidative stress in OSA patients and experimental animals exposed to IH.

Evidence for Oxidative Stress in OSA Patients

In earlier studies, oxidative stress was assessed by monitoring either free radical-induced formation of diene-conjugate of LDL [121] or hydrogen peroxide-induced lipid peroxidation (by measuring malondialdehyde levels), red blood cell fragility (as an indicator of cell membrane oxidative stress), or total cellular sulfhydryl (thiols) levels [83]. The results from these studies show no significant difference in the levels of these markers of oxidative stress among OSA patients and healthy humans.

In more recent studies, investigators using a larger sample size and a different marker of oxidative stress are able to detect increased oxidative stress in OSA patients

compared to control subjects. For instance, Christou et al. [24] report a higher levels of diacron-reactive oxygen metabolites in the blood of OSA patients ($n=21$) than in control subjects ($n=5$). Likewise, formation of thiobarbituric acid-reactive substance (T-BARS) is higher in patients with severe OSA (59 apneas/h; $n=14$) than in healthy subjects ($n=13$) [10]. In a larger study involving 114 OSA patients, morning levels of T-BARS and peroxides are found to be significantly higher in OSA patients, with or without cardiovascular disease, than in controls [66, 67]. A recent study by Yamauchi et al. [124] demonstrated that urinary 8-hydroxy-2′-deoxyguanosine excretion was significantly higher in patients with severe OSA ($n=58$) compared with control subjects ($n=70$). Also, [33] report an increase in the production of ROS in leukocytes isolated from sleep apnea patients compared to healthy subjects.

A reduction in antioxidant capacity also leads to oxidative stress. Analysis of the blood of severe OSA patients (apnea/hypopnea index of >20; $n=14$) and healthy humans ($n=5$) using the Trolox equivalent antioxidant capacity assay show decreased total antioxidant status in OSA patients [25].

Although results from studies described above indicate the occurrence of oxidative stress in systemic circulation of OSA patients, several other studies report no detectable changes in the levels of lipid peroxidation in OSA patients [2, 83, 117, 121]. These conflicting results may be explained by the small size of the OSA patients and healthy subjects, possible presence of comorbidities and/or medications in OSA patients, the absence of control subjects matched closely for body mass index and obesity, the presence of undiagnosed OSA in control populations, the timing of oxidative stress measurements, and proper handling of the samples to avoid further oxidation [117]. Also, environmental, dietary, and genetic factors can also influence the levels of oxidative stress in humans. It is therefore necessary to include large sample sizes and use highly sensitive and reliable techniques to evaluate accurately the oxidative stress in humans. On the basis of results from large population-based studies, it is safe to conclude that a subset of patients with severe OSA exhibit oxidative stress.

Few studies have investigated the effects of antioxidants on endothelial dysfunction in OSA patients. Either vitamin C infusion [44] or allopurinol treatment [34] seems to improve endothelial dysfunction in patients with severe OSA. Only a limited number of studies have investigated the effect of CPAP on oxidative stress and the results are inconclusive [9, 21, 23, 117, 118].

Oxidative Stress in Animal Models of IH

Evidence for IH-Induced ROS Generation

The possibility of ROS generation in experimental animals during the reoxygenation phase of IH is investigated by several groups. Studies in rats assessed ROS generation by using one or more of the following markers of oxidative stress: levels of T-BARS as index of lipid peroxidation; aconitase activity, which is sensitive to and inhibited by ROS [40]; and isoprostane levels [20]. The results from these studies show that IH

facilitates ROS generation in the central and peripheral nervous systems implicated in the control of autonomic and cardiovascular function including the carotid body, the primary peripheral chemoreceptor that detects arterial levels of oxygen [88], carotid sinus region, the site of carotid baroreceptors [87], adrenal medulla, a constituent of the sympathetic nervous system contributing to circulating catecholamines [63, 98, 115], brainstem, regulating sympathetic activity, and cardiovascular function [99, 100, 105]. Also increased ROS generation is observed in rat brain region associated with spatial learning [17, 102] and in left ventricles of rats exposed to chronic IH [22]. Studies in mice also reveal increased ROS generation in brain cortex [122] and brain regions associated with sleep-wake regulation [120]. Unlike IH, sleep deprivation alone has no significant effect on ROS formation in the brain, liver, and skeletal muscle of rats [43]. Therefore, IH, rather than sleep fragmentation, induces oxidative stress in OSA associated with recurrent apneas.

Role of ROS in IH-Induced Responses

To assess the functional significance of increased ROS levels, the effects of antioxidants on autonomic and cardiovascular functions are assessed. Rats are treated daily with manganese (III) tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride (MnTMPyP; 5 mg/kg, IP), a membrane-permeable superoxide dismutase (SOD) mimetic, prior to a 8 h regimen of IH exposure for 10 days. MnTMPyP treatment is found to be highly effective in preventing IH-evoked heightened hypoxic sensitivity, induction of sensory long-term facilitation, and augmented HVR in adults [88, 89] and in neonates [85], as well as depressed baroreflex function [87]. Antioxidant also abolishes the enhanced catecholamine secretion from the AM of IH-treated neonatal [113] and adult rats [63] and mice [64]. Furthermore, antioxidants prevent serine phosphorylation of TH, increases in enzyme activities of TH, protein kinase A, ERK and CaMKII as well as the inhibition of PP2A elicited by IH in the rat brainstem [99]. Collectively, these findings support a critical role for ROS in chemo- and baroreflex and adrenal medullary and brainstem responses to IH.

Furthermore, learning deficits induced by IH is attenuated by the antioxidant PNU-101033E [102]. ROS production and neuronal death associated with IH exposure are absent in transgenic mice overexpressing Cu/Zn-SOD, an antioxidant enzyme [122]. Likewise, pretreatment with green tea catechin ployphenols attenuates IH-induced increase in malondialdehyde levels, an index of ROS generation, as well as neurocognitive deficits associated with IH [17].

Sources of ROS Generation During IH

The IH-evoked ROS elevation could be due to either an increase in ROS synthesis via upregulation of ROS-generating enzymes or a reduction in ROS degradation via downregulation of antioxidant enzymes. NADPH oxidase (Nox) family, xanthine

oxidase, and uncoupled eNOS contribute to ROS production, whereas ROS degradation is mediated by antioxidant enzymes that include SOD-1 and 2, catalase, and glutathione peroxidase. ROS is also generated via inhibition of complex I and III of the mitochondrial electron transport chain (ETC; [3]). Regarding the effects of antioxidants on IH, a recent study by Nanduri et al. [76] provides some evidence for IH-induced downregulation of antioxidant enzymes. They report a reduction in SOD-2 mRNA and SOD-2 enzyme activity in the AM of rats exposed to IH. These results suggest that the increased ROS levels caused by IH may involve downregulation of antioxidant enzymes.

Evidence for the Role of Nox

Available evidence suggests that the elevated ROS induced by IH is in part due to increased ROS generation via activation of Nox family of enzymes. For instance, Peng et al. [86] report a 12-fold increase in Nox enzyme activity which is accompanied with an increase in Nox2 mRNA in the carotid body of rats treated with IH. The contribution of Nox2 is supported by studies in Nox2 knockout mice showing absence of IH-induced ROS and functional changes in the carotid body [86]. IH also upregulates Nox2 mRNA and increases Nox activity in the AM of neonatal rats exposed to IH [115]. Interestingly, these authors report that Nox inhibitors prevent acute hypoxia-evoked exaggerated catecholamine secretion as well as increase in $[Ca^{2+}]_i$ in the AM of IH-treated neonatal rats. In addition to Nox2, IH also upregulates Nox4 mRNA in the carotid body and AM; however, the functional significance of Nox4 in IH-induced response remains to be investigated. Burckhardt et al. [17] also report an increase in protein levels of p47phox, a Nox subunit, in hippocampal CA1 neuron of rats after 14 days of IH exposure. A similar increase in Nox subunit expression is also reported in sleep-awake brain region of IH-treated mice [120, 128]. Collectively, these studies suggest that Nox is one of the major sources of ROS production in experimental animals treated with IH.

Evidence for the Role of Mitochondrial Electron Transport Chain

In addition to oxidases, mitochondrial ETC is another major source of ROS [3]. Peng et al. [88] show that mitochondrial aconitase activity is lower in carotid bodies from IH-treated rats compared to normoxia exposed controls suggesting generation of ROS in the mitochondria. Studies by Schumacker and his coworkers [47] suggest that continuous hypoxia induces increased ROS production via complex III of the ETC. However, studies in experimental models show that IH selectively inhibits complex I but not III in the rat carotid body [88] and cell cultures [125]. These studies imply that unlike continuous hypoxia, mitochondrial complex I is one of the sources of ROS generation during IH.

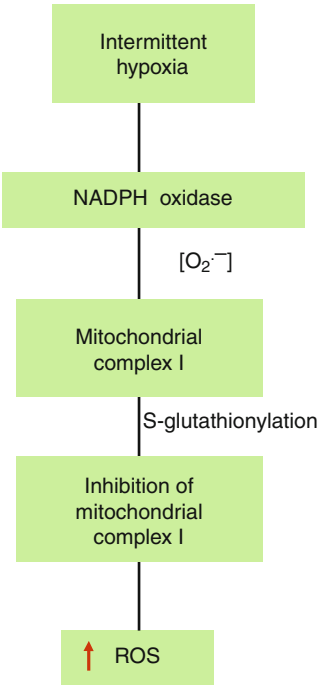

Fig. 4.3 Mechanism of mitochondrial complex I inhibition by intermittent hypoxia

Mechanisms of Mitochondrial Complex I Inhibition by IH

How does IH inhibit mitochondrial complex I activity? To test whether Nox-derived ROS mediate mitochondrial complex I inhibition, Khan et al. [60] simultaneously analyzed the activities of Nox and complex I in rat pheochromocytoma (PC)-12 cell cultures exposed to 60 cycles of IH (1.5 % O_2 for 30 s followed by 20 % O_2 for 5 min at 37 °C). Their results show that (1) IH, in a stimulus-dependent manner, increases Nox activity with a concomitant inhibition of complex I activity; (2) Nox activity returns to baseline values after 3 h of reoxygenation, whereas inhibition of complex I persists even after 24 h of reoxygenation; and (3) either pharmacological inhibition of Nox or siRNA silencing of Nox2 but not Nox4 abolishes IH-induced inhibition of complex I. These results suggest that Nox-derived ROS inhibits complex I in IH-exposed cells. Khan et al. [60] further show that Nox-derived ROS via mobilization of cytosolic Ca^{2+} to mitochondria facilitate S-glutathionylation of 75- and 50-kDa subunits of complex I. Thus, complex I inhibition by IH occurs via Nox-ROS-Ca^{2+}-dependent posttranslational modification of complex I involving S-glutathionylation reaction. A similar S-glutathionylation reaction is also responsible for the activation of ryanodine receptor-2 which mediates Ca^{2+} mobilization needed for catecholamine secretion in the AM of neonatal rats exposed to IH [115]. Taken together, these findings provide evidence for functional interaction between Nox2 and mitochondrial complex I which generates a sustained increase in ROS levels via mechanism involving ROS-induced ROS generation (Fig. 4.3).

Functional Significance of Mitochondrial ROS

The role of ROS generated by Nox and mitochondria in the elevation of blood pressure by IH is investigated using apocynin (10 mg/kg; IP), a Nox inhibitor and mitochondria-targeted tempol (mito-tempol; 10 mg/kg; IP), which inhibits oxidant formation from mitochondria each day before 8 h regimen of IH exposure for 10 days [60]. Rats treated with vehicle and then exposed to IH for 10 days serve as controls. In conscious rats, post-IH blood pressure is monitored after 1 h (early phase) and 15 h (late phase). In vehicle-treated IH rats, MAP remains elevated both in the early and late phases, whereas these responses are absent in apocynin-treated IH rats. Notably, mito-tempol treatment selectively abolishes IH-evoked elevation in MAP in the late phase without affecting the early phase suggesting mitochondrial-derived ROS contribute to sustained elevation in blood pressure.

Molecular Mechanisms of ROS Generation During IH

Available evidence suggests that transcriptional mechanisms involving hypoxia-inducible factors, HIF-1α and HIF-2α, contribute to sustained oxidative stress elicited by IH. A detailed account of these mechanisms can be found in recent reviews [94, 96]. IH has been shown to increase HIF-1α and decrease HIF-2α protein expression in a ROS-dependent manner [76, 77, 126]. HIF-1α upregulation by IH involves both stabilization and protein synthesis via mTOR pathway [126] and appears to be linked to increased prooxidants (e.g., Nox). The downregulation of HIF-2α induced by IH occurs via calpain-mediated protein degradation and is coupled to transcriptional downregulation of antioxidant enzymes (e.g., SOD-2; [76]). HIF-1α heterozygous mice do not show IH-induced hypertension. On the other hand, blocking IH-induced downregulation of HIF-2α in rats with calpain inhibitor, ALLM, prevents hypertension caused by IH. These findings suggest a novel, hitherto unrecognized roles for HIFs in redox regulation and identify HIFs as one of the major molecular mechanisms underlying IH-induced oxidative stress and hypertension.

Conclusion

In summary, studies in OSA patients and experimental animals exposed to IH show that ROS generated via functional interaction between Nox and mitochondrial ETC contribute to autonomic and cardiovascular abnormalities. These ROS-induced effects seem to involve a complex interplay of redox-sensitive transcription factors, vasoactive hormones/transmitters, membrane receptors, ion channels, and changes in Ca^{2+}-handling (Fig. 4.4). Future studies are needed to define the mechanisms by which IH activates Nox, to delineate potential roles of other cytosolic oxidases in IH-induced ROS generation, and to identify additional cellular targets of ROS under the setting of recurrent apneas.

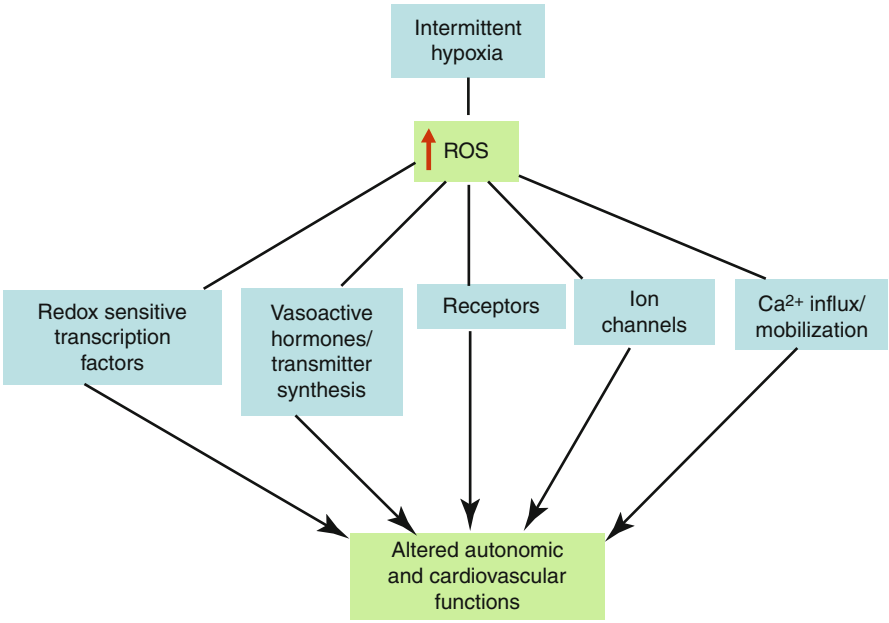

Fig. 4.4 Interaction of ROS with various cellular targets eliciting autonomic and cardiovascular abnormalities during intermittent hypoxia

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Chapter 5
Mitochondrial Excitation-Energy Coupling in Airway Smooth Muscle

Niccole Schaible, Philippe Delmotte, and Gary C. Sieck

Abstract Force generation and contraction of human airway smooth muscle (ASM) involves both an increase in intracellular Ca^{2+} and an increase in the demand for energy in the form of ATP. Excitation-contraction coupling (ECC) represents a cascade of events that connects the initiating signal, an elevation of cytosolic Ca^{2+}($[Ca^{2+}]_{cyt}$), with the ensuing ATP-consuming mechanical work. Mitochondria play a vital role in this overall process by producing ATP. Moreover, mitochondria also possess the ability to sense $[Ca^{2+}]_{cyt}$ through a coupled increase in mitochondrial Ca^{2+}($[Ca^{2+}]_{mito}$). In fact, an increase in $[Ca^{2+}]_{mito}$ leads to an increase in ATP production. Thus, the linkage between $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mito}$ may reflect an "excitation-energy coupling" in ASM that mirrors the energy demand that results from excitation-contraction coupling. The result, in ASM, is the maintenance of an energy supply despite transient and/or sustained energetic demands – an energy homeostasis.

Keywords Excitation-contraction coupling • Metabolism • Ca^{2+} signaling • Homeostasis • Energetics

Introduction

Asthma affects more than 20 million people in the USA (~10 % of the population) and more than 300 million people worldwide [35]. The incidence and severity of asthma is increasing, with a 47 % increase in death rate associated with asthma over the past 10 years. Asthma is characterized by airway smooth muscle (ASM) hyperresponsiveness which is most likely a reflection of changes in the contractile response.

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Mitochondria play a vital role in the overall contractile response (which consumes ATP) by producing ATP. The balance between energy demand (ATP hydrolysis) and energy supply (ATP production) determines cytosolic ATP levels. However, it has been observed in smooth muscle that ATP levels do not measurably fluctuate during normal contractile responses [62]. Therefore, increased energy demand of the contractile response must be matched by equivalent increases in energy supply. A way this matching might occur is through Ca^{2+} since both contractile and energetic mechanisms are regulated by Ca^{2+}. On one hand, increases in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) signal the contractile response through a process known as excitation-contraction coupling (ECC). On the other hand, there are metabolic processes, including ATP production within the mitochondria, that are stimulated by an elevation of mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_{mito}$) [23, 26, 36, 37]. By virtue of this link between $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mito}$ and ATP, mitochondria may perform an "excitation-energy coupling," which mirrors excitation-contraction coupling. The result, in ASM, is the maintenance of a persistent energy supply despite transient or sustained energetic demands – an energy homeostasis.

The ASM contractile response involves two associated components: (1) mechanical force and (2) energy demand in the form of ATP hydrolysis. While this is also true for skeletal muscle, there are significant differences between smooth and skeletal muscle in terms of mechanics and energetics during contraction. For example, smooth muscle is able to sustain stable force generation over a wide range of lengths and for extended periods of time in comparison to skeletal muscle. The differences in smooth muscle versus skeletal muscle contractile responses are likely reflections of their disparate physiological roles, which favor efficiency versus power, respectively.

In this review, the role of intracellular Ca^{2+} in coordinating the energy demand of mechanical work with the energy supplied by mitochondria in ASM will be systematically examined. The overall conceptual framework is described in two main parts. The first part explores the role of $[Ca^{2+}]_{cyt}$ in excitation-contraction coupling and energy demand. The second part explores the role of $[Ca^{2+}]_{mito}$ in excitation-energy coupling and energy supply.

A Conceptual Framework for the Role of $[Ca^{2+}]_{cyt}$ in Excitation-Contraction Coupling in ASM

Spatial/Temporal Encoding of a Contractile Stimulus by $[Ca^{2+}]_{cyt}$

Contraction of ASM begins with an extracellular cue that triggers an increase in $[Ca^{2+}]_{cyt}$. This section will not discuss the pathways specific to a particular agonist, but rather focus on the main mechanisms involved in the regulation of $[Ca^{2+}]_{cyt}$. Similarly, differences in Ca^{2+} signaling have been found between species and

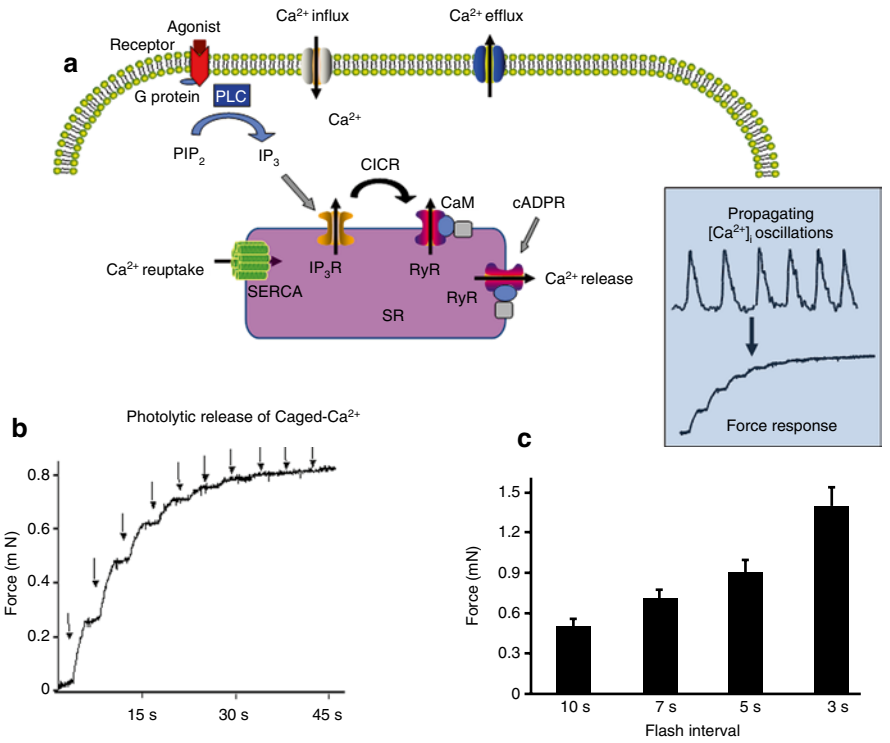

Fig. 5.1 (**a**) ASM model highlighting main components of the encoding of a force response by $[Ca^{2+}]_{cyt}$ where propagating $[Ca^{2+}]_{cyt}$ oscillations give rise to a sustained force response. (**b**) Demonstration of the sustained response of force due to a sequence of $[Ca^{2+}]_{cyt}$ increases by flash photolytic release of caged Ca^{2+}. (**c**) Decreasing the flash interval time of photolytic release of Ca^{2+} resulted in a correlated increase in level of steady-state force

between large and small airways but will not be discussed in this section [23, 89]. It is assumed that the same underlying mechanisms are present in all ASM, yet their relative contribution may change depending on type.

Changes in $[Ca^{2+}]_{cyt}$ are due to Ca^{2+} flux between the extracellular space, the cytosol, and the sarcoplasmic reticulum (SR). While controversial, it has been shown in ASM that neither plasma membrane (PM) Ca^{2+} flux nor PM depolarization is required for the immediate ECC response [61, 79]. This is a significant difference with skeletal muscle which rely on PM Ca^{2+} influx for the ECC response [93]. Although PM Ca^{2+} channels are not required during the immediate $[Ca^{2+}]_{cyt}$ response to agonist stimulation in ASM, they have an important role in intracellular Ca^{2+} homeostasis [79, 109].

Agonists binding to phospholipase C-coupled receptors on the PM lead to the production of inositol 1,4,5-trisphosphate (IP_3) which binds and activates inositol 1,4,5-trisphosphate receptors (IP_3R) to release Ca^{2+} from the SR (Fig. 5.1a) [31].

The increase in $[Ca^{2+}]_{cyt}$ activates nearby clusters of IP_3Rs and ryanodine receptors (RyR) – an amplification and propagation process called Ca^{2+}-induced Ca^{2+} release (CICR). The kinetics of IP_3R and RyR are modulated by $[Ca^{2+}]_{cyt}$ levels where $[Ca^{2+}]_{cyt}$ has the potential for either positive or negative feedback – the relationship of channel open probability to $[Ca^{2+}]_{cyt}$ is represented by a bell-shaped curve. The open probability of the RyR channel can also be modulated by cADPR [60, 80, 108] as wells as ryanodine, caffeine, and FK506-binding proteins [112]. The SR possesses a Ca^{2+} uptake pump, the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA), which continually opposes the Ca^{2+} efflux action of open IP_3R and RyR channels to refill the SR and bring $[Ca^{2+}]_{cyt}$ back to basal levels. The dynamic interplay between SR Ca^{2+} release via IP_3R or RyR channels and SR Ca^{2+} uptake via SERCA, in ASM cells, leads to an increase in $[Ca^{2+}]_{cyt}$ in the form of propagating Ca^{2+} oscillations at a frequency that ranges from ~5–40 min^{-1} and an amplitude that ranges from ~100–800 nM of $[Ca^{2+}]_{cyt}$ [79, 81].

The $[Ca^{2+}]_{cyt}$ response to agonist stimulation, which oscillates, travels as a wave which radiates out from a discrete site. The "Ca^{2+} wave" usually originates from the same site within an ASM cell after repeated stimulus and also maintains the same wave front direction across the cytosol [73, 79, 81]. Also, before stimulation, spontaneous yet spatially isolated $[Ca^{2+}]_{cyt}$ transients, known as "Ca^{2+} sparks," can be observed at random locations within the ASM cell [73]. Ca^{2+} sparks are rapid with an estimated rise time of ~30–90 ms and fall time of ~60–300 ms [73]. Ca^{2+} sparks are attributed to the activation kinetics of RyR channels [72, 73, 96]. The Ca^{2+} spark, a stochastic event, is the most elementary of Ca^{2+} release events [72, 73, 96].

The frequency as well as propagation velocity of $[Ca^{2+}]_{cyt}$ oscillations increases with agonist concentration [79, 81] and may be reflections of RyR-mediated CICR sensitivity [81]. Frequency modulation of $[Ca^{2+}]_{cyt}$ during agonist stimulation is proposed as a mode of control of the contractile response (Fig. 5.1) [79, 81].

Excitation-Contraction Coupling Time Frame

When ASM cells are exposed to 1 μM ACh, an elevation in $[Ca^{2+}]_{cyt}$ is induced, yet evidence of muscle shortening is delayed by several hundred milliseconds [94]. This time delay reflects the time required for a cascade of events (Fig. 5.2) that can be summarized into four main steps: (1) mobilization of CaM, (2) binding of Ca^{2+} to CaM and subsequent activation of MLCK, (3) phosphorylation of MLC_{20}, and finally (4) crossbridge recruitment. The relative contribution of each the four delays have been quantified in ASM by measuring the response to rapid (virtually instantaneous) release of caged compounds by flash photolysis [94]. The results of this study indicate that mobilization of CaM is the most significant delay, while the delay due to phosphorylation of MLC_{20} and crossbridge recruitment is minor [94].

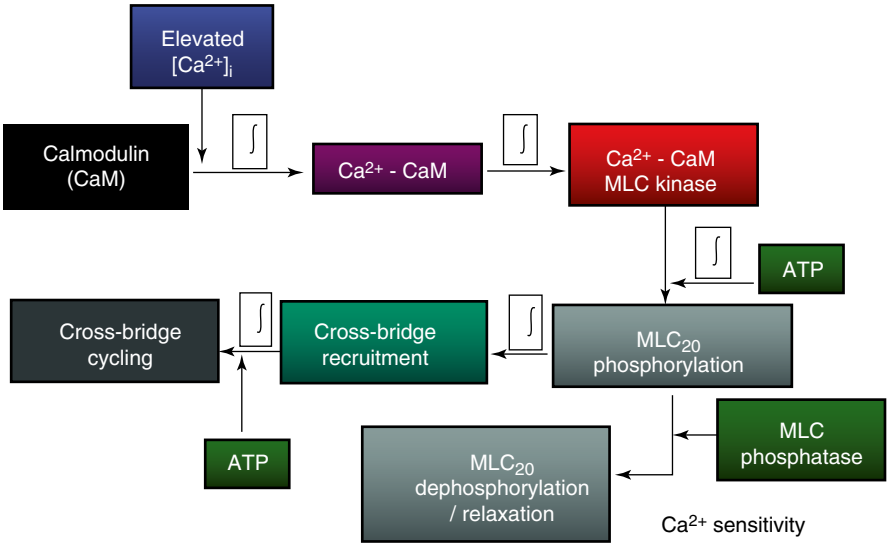

Fig. 5.2 Excitation-contraction coupling in airway smooth muscle cells indicating main temporal delays [93]. In a previous study [94], the rate-limiting step was found to be the delay between CaM and Ca^{2+}-CaM

Mechanical Function and ATP Hydrolysis

The primary role of muscle (skeletal muscle or smooth muscle) is to perform mechanical work through contraction force generation. To perform mechanical work, muscles require chemical energy in the form of ATP hydrolysis. The relationship between muscle work and ATP hydrolysis is not as well defined in smooth muscle compared to skeletal muscle. Therefore, to provide a conceptual framework for the dynamic range of energy demand in smooth muscle, the relationship between mechanical work and energy consumption in skeletal muscle will serve as a point of reference.

The molecular mechanism responsible for contraction in smooth muscle, like all muscle, is the contractile protein apparatus, actin and myosin, conferred by its dual ability to enzymatically harvest chemical energy and, subsequently, perform mechanical work. Chemical energy is harvested via ATP hydrolysis by the actin-activated myosin ATPase (actomyosin ATPase). The energy released by hydrolysis of ATP is coupled to the intrinsic force development by cyclic attachment and detachment of myosin heads to actin (crossbridge cycling).

The intrinsic structural unit that underlies force generation and contraction in skeletal muscle is the actin-myosin crossbridge. Andrew Huxley originally proposed this relationship in a simple model where crossbridge cycling reflected the repetitive transition between two possible mechanical states: (1) myosin attached to actin and generating force and (2) myosin detached from actin [53, 54]. In this

model, the hydrolysis of ATP occurs during the transition between attached and detached states. Thus, the Huxley model provides a simplified conceptual framework for both excitation-contraction coupling and excitation-energy coupling.

Skeletal muscle actin-myosin orientation is precisely ordered into units of repeating architecture referred to as sarcomeres – which are important context for application of the Huxley model to skeletal muscle. In brief, the sarcomere is made up of an interlacing, crystalline formation of thick filaments (myosin) and thin filaments (actin). The boundary of a sarcomere is formed by a dense *Z*-disk or *Z*-line, which fasten the actin filaments. Actin filaments extend from *Z*-line to the midline of the sarcomere. The myosin filaments, centered at the midline of the sarcomere, lie between and parallel to actin filaments such that there are six actin filaments surrounding each myosin filament. During muscle contraction, crossbridge cycling within the overlapping regions of the actin and myosin filaments within a half-sarcomere pulls its associated *Z*-line towards the midline. Thus, the force and contraction vectors are determined by actin-myosin crossbridges within a half-sarcomere. For more details, see review by [93].

The Huxley model has been expanded to a quantitative form by [8, 9]. To summarize, the fraction of strongly bound crossbridges in the force-generating state (Huxley's two-state model) is represented as "α_{fs}". A strongly bound crossbridge is able to supply a unit of force, which is approximated as an averaged quantity of force per crossbridge and designated by "f". The maximal number of myosin heads available for crossbridges formation within a half-sarcomere is represented as "n". The combination of these factors determines force (F) generated by a muscle fiber as shown in Eq. 5.1.

$$F = n \; f \; \alpha_{fs} \tag{5.1}$$

The fraction of crossbridges in a force-generating state (α_{fs}) is a function of the rates of transition between the two mechanical/structural states of the Huxley model as shown in Eq. 5.2 below, where "f_{app}" is the apparent rate constant for transition to the attached state, and "g_{app}" is the apparent rate constant for transition to the detached state.

$$\alpha_{fs} = f_{app} / \left(f_{app} + g_{app} \right) \tag{5.2}$$

Relationships that characterize skeletal muscle function (force vs. Ca^{2+}, force vs. length, and force vs. cross-sectional area) can be accounted for by this model. The parameter α_{fs} can be increased by an increase in $[Ca^{2+}]_{cyt}$ which promotes transition to the attached, force-generating state of crossbridges (force-Ca^{2+} relationship). Muscle length, which in turn defines sarcomere length and the extent of actin-myosin filament overlap, also affects α_{fs} without an effect on n (force-length relationship). Finally, fiber cross-sectional area reflects the number of sarcomeres in parallel and, thus, myosin content per half-sarcomere (myosin content determines n) [95].

Using this conceptual framework, a relationship can also be described for ATP consumption assuming a stoichiometry of 1 mole ATP consumed per crossbridge detachment. This relationship is shown in Eq. 5.3 where "b" represents the number of half-sarcomeres within the fiber.

$$\text{ATPase} = nbg_{\text{app}}\alpha_{\text{fs}} \tag{5.3}$$

The load opposing the intrinsic crossbridge-induced sarcomeric contraction slows crossbridge cycling rate due to its effect on $g_{\text{app.}}$ From Eq. 5.2, this slowing of g_{app} has the effect of increasing α_{fs} and thereby increasing force generation to the point where there is no contraction (isometric force). The rate of contraction (shortening velocity) depends inversely on external load imposed on the sarcomere and therefore determines the force (or load)-velocity relationship. From Eq. 5.3, the rate of ATP hydrolysis ("ATPase" in Eq. 5.3) also depends on g_{app}; thus, external load and intrinsic force generation opposing the load will influence the rate of ATP hydrolysis. The direct relationship between heat (reflecting energy consumption) and power output of muscle due to contraction (the product of force and shortening velocity) is known as the Fenn effect and was first described in 1923 [29]. Thus, the maximum rate of ATP hydrolysis occurs at peak power output in skeletal muscle [93, 95, 97].

In skeletal muscle fibers, isometric force generation at any given level of Ca^{2+} activation is accompanied by a sustained rate of ATP hydrolysis as described by Eq. 5.3 [95] .Thus, the energy cost of force generation does not change during a given level of isometric activation. In contrast, during sustained isometric force generation of smooth muscle, the rate of ATP hydrolysis decreases with time even as force is sustained [25, 48, 104]. This indicates that in smooth muscle, the energy cost of producing force decreases with time – sustained smooth muscle force generation becomes more energy efficient. The energy cost of force generation in smooth muscle can be explained by the Huxley model with consideration of the differences between smooth and skeletal muscle mechanics. For example, there are key differences in actin-myosin alignment, actin-myosin remodeling, and Ca^{2+} regulation of crossbridge recruitment. Each of these factors will be considered in the next sections.

Actin-Myosin Alignment

Monomeric myosin is assembled into polymers or thick filaments in a nonhelical, side-polar configuration in smooth muscle [50, 110], while in skeletal muscle myosin filaments form a helical, bipolar configuration (Fig. 5.3). The bipolar arrangement forms a "bare area" at the middle of the myosin thick filament. This bare area and the steric constraints of the sarcomeric structure impose limits on muscle length in skeletal muscle that do not exist in smooth muscle. The side-polar arrangement of actin-myosin filaments in smooth muscle allows a significantly greater length range [91, 110].

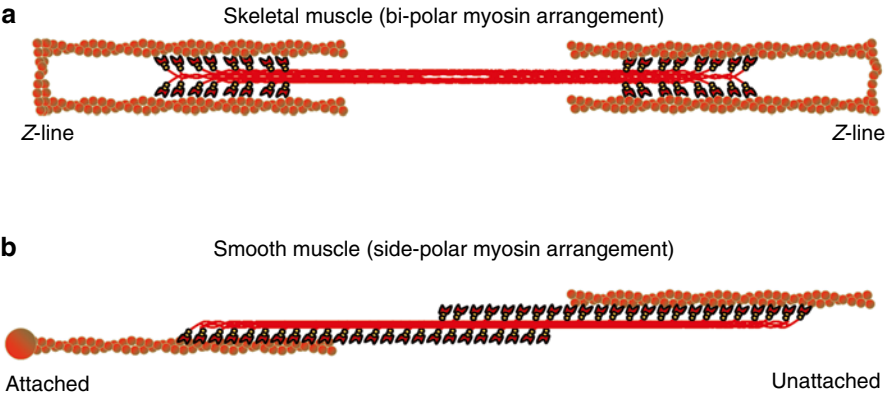

Fig. 5.3 (**a**) Schematic representation of the bipolar arrangement of myosin in skeletal muscle and (**b**) the side-polar arrangement of myosin in smooth muscle. The interaction of myosin (*red*) with actin (*brown*) is compared between both muscle types where in skeletal muscle (**a**) actin-myosin structure is fastened by *Z*-lines whereas in smooth muscle (**b**) the actin-myosin lattice structure is dynamic and can potentially be fastened to dense bodies or membrane-associated dense plaques (represented by the attached end) and/or potentially unloaded (Represented by the unattached end)

Actin-Myosin Remodeling

In both smooth and skeletal muscle, myosin thick filaments interact with actin thin filaments to form crossbridges. However, in skeletal muscle, actin filaments are anchored in a regular fashion by the *Z*-disks of the sarcomere, whereas in smooth muscle, actin filaments are clustered into regions called dense bodies or membrane-associated dense plaques (Fig. 5.3) [39, 93]. The dense bodies fasten the side-polar actin-myosin lattice and allow force transmission [50, 63] – analogous to the *Z*-disk in skeletal muscle sarcomeres [39]. However, the internal arrangement of the contractile apparatus (actin-myosin filaments, dense bodies, and dense plaques) in smooth muscle is not regularly ordered and may undergo remodeling during the contractile response (see reviews [41, 91, 113]). Thus, although external isometric conditions may be maintained, dynamic internal remodeling may change the length and overlap relationships between actin and myosin filaments. From Eq. 5.1, force in skeletal muscle is dependent on the number of myosin heads (n) and the fraction of myosin heads forming crossbridges (α_{fs}). Actin and myosin filament remodeling in smooth muscle will affect both n and α_{fs}. In addition, actin and myosin filament remodeling will result in changes in internal loading that affect crossbridge cycling rate (g_{app} from Eq. 5.2), α_{fs} (Eq. 5.1), and the rate of ATP hydrolysis (Eq. 5.3).

There is evidence of changes in actin polymerization during Ca^{2+} activation and force generation in smooth muscle [40, 49, 68]. Actin exists in two distinct but interchangeable forms: polymeric, filamentous actin (F-actin) and monomeric actin (G-actin) [41, 58, 68]. During contraction, there is a decrease in G-actin content in

ASM [68]. Pharmacological inhibition of actin polymerization reduces isometric force generation in smooth muscle [40, 58, 68], which suggests that actin polymerization plays an important role in the contractile response. In addition to actin polymerization, there is a reorganization of the contractile network through myosin polymerization [1, 55, 64, 65, 91] that changes the length of the myosin filaments and thus the number of myosin heads that can potentially form crossbridges (n in Eq. 5.1). Filamentous actin is also coupled to the extracellular matrix (ECM) by focal adhesion complexes (membrane-bound dense plaques) [40]. Focal adhesion complexes are comprised of a scaffold of structural and signaling proteins, including mechanosensitive transmembrane integrins [41, 113]. The location of focal adhesion complexes around the PM is dynamically regulated during contraction [3, 32]. In other cell types which are motile, focal adhesions orchestrate actin polymerization to cause cell movement [41, 113]. A similar process may occur in the ASM where focal adhesions recruit actin polymerization (increasing internal loading) according to local mechanical stimuli.

In addition to internal aspects of a contractile stimulus, the ECM transmits mechanical loads (external load) onto the focal adhesions. This external load in vivo is dynamic due to inflation and deflation of the lung [40, 41, 113]. Dynamic changes in external loading may stimulate changes (perhaps via integrin-mediated pathways) in actin-myosin filament architecture. The actin-myosin architecture at any given moment is therefore a function of the history of mechanical stimuli [39, 49, 64] and influences internal loading of crossbridges.

Mechanisms of Ca^{2+}-Induced Activation

In response to an elevation of $[Ca^{2+}]_{cyt,}$ crossbridge recruitment in smooth muscle is myosin-activated, whereas in skeletal muscle Ca^{2+} regulation of crossbridge recruitment is actin-regulated. Skeletal muscle actin is associated with other proteins, troponin and tropomyosin, that regulate exposure of the myosin-binding site on actin. In skeletal muscle, with an increase in $[Ca^{2+}]_{cyt}$, troponin C binds Ca^{2+}, which induces a conformational change in troponin I and troponin T followed by transposition of tropomyosin uncovering the myosin-binding site on the actin filament. With the myosin-binding site uncovered, crossbridge recruitment and cycling proceeds [93].

In contrast, in smooth muscle, Ca^{2+}-dependent regulation of crossbridge recruitment requires phosphorylation of the regulatory domain of myosin (MLC_{20}) by myosin light-chain kinase (MLCK) (Fig. 5.2). MLCK activity is stimulated by an increase in $[Ca^{2+}]_{cyt}$ through the binding of Ca^{2+} to calmodulin (CaM). The extent of MLC_{20} phosphorylation is also regulated by myosin light-chain phosphatase (MLCP), which is not Ca^{2+}-dependent but is regulated by a Rho kinase pathway [98]. As a consequence, the phosphorylation of MLC_{20} reflects the balance between MLCK and MLCP activities and, therefore, Ca^{2+}-dependent and independent (i.e., Ca^{2+} sensitivity) mechanisms.

"Latch-Bridge" Hypothesis

Smooth muscle force generation and contraction is generally initiated by a transient elevation of $[Ca^{2+}]_{cyt}$ with an associated transient increase in MLC_{20} phosphorylation. Yet, force generation in smooth muscle can be maintained at a constant level despite transients in $[Ca^{2+}]_{cyt}$ or MLC_{20} phosphorylation. Therefore, there are two distinct phases of force generation in smooth muscle initiated by a transient elevation of $[Ca^{2+}]_{cyt}$: (1) the initial increase in both force and MLC_{20} phosphorylation and (2) the maintenance of force despite decreasing $[Ca^{2+}]_{cyt}$ and MLC_{20} phosphorylation. This second phase of smooth muscle activation is also associated with a slowing of shortening velocity (crossbridge cycling rate) and has been termed the "latch state" [44, 69]. According to this hypothesis, a unique crossbridge state – the "latch-bridge" – is formed when the myosin head of an attached crossbridge (due to prior MLC_{20} phosphorylation) is then dephosphorylated. These "latch-bridges" cycle at a slower rate with an increased duty cycle (amount of time attached vs. detached) and, therefore, maintain force at a lower level of MLC_{20} phosphorylation or $[Ca^{2+}]_{cyt}$ reflecting increased Ca^{2+} sensitivity of force generation. The main assumption of the latch-bridge model is that the extent of MLC_{20} phosphorylation determines the "latch" or energetic state of smooth muscle force generation [44].

In a Triton X-100 permeabilized ASM preparation [57] directly tested the latch-bridge model by measuring changes in isometric force, MLC_{20} phosphorylation, and the rate of ATP hydrolysis during maximum Ca^{2+} activation (10 μM Ca^{2+}). With maximum Ca^{2+} activation, force increased with a concomitant increase in both MLC_{20} phosphorylation and the rate of ATP hydrolysis. As Ca^{2+} activation remained constant, isometric force was sustained, as was the level of MLC_{20} phosphorylation. However, after peaking, the rate of ATP hydrolysis decreased while force and the level of MLC_{20} phosphorylation were sustained, reflecting a reduction in energy cost, consistent with a "latch" state, but without a change in MLC_{20} phosphorylation [57]. To further test the latch-bridge hypothesis, thiophosphorylation (accomplished by incubation with ATPγS) was used to prevent dephosphorylation of MLC_{20} [57]. After thiophosphorylation, the dynamic change in the rate of ATP hydrolysis with sustained Ca^{2+} activation was unaffected, also indicating that there is a slowing of crossbridge cycling rate that is independent of any change in the level of MLC_{20} phosphorylation. This indicates that the level of MLC_{20} phosphorylation can be dissociated from the rate of ATP hydrolysis and that the "latch-bridge" as proposed does not actually exist.

"Internal Loading" Hypothesis

An alternate "internal loading" hypothesis may explain the decline in the rate of ATP hydrolysis (or energy cost) in ASM during sustained Ca^{2+} activation [58]. In this hypothesis, internal loading on crossbridges in ASM is not fixed under

isometric conditions, but can change by dynamic cytoskeletal remodeling. It has been demonstrated in ASM that inhibition of actin reorganization with phalloidin (which binds F-actin, preventing its depolymerization) significantly lessens the decline in the rate of ATP hydrolysis during sustained Ca^{2+} activation, thereby increasing the energy cost of force generation [58]. The "internal loading" model can be broken down into two phases: (1) an initial phase when crossbridges are recruited by MLC_{20} phosphorylation (increasing α_{fs} with an associated increase in the rate of ATP hydrolysis as predicted by Eqs. 5.3 and 5.2) and (2) a delayed phase where there is an increase in internal loading due to cytoskeletal remodeling, with an associated slowing g_{app} and decrease in the rate of ATP hydrolysis (Eq. 5.3). During the initial phase of Ca^{2+} activation, a portion of the actin-myosin filaments are not fully attached to the internal cytoskeletal structure, resulting in lower internal loading, faster g_{app}, and a high rate of ATP hydrolysis. Subsequently, with cytoskeletal remodeling, there is an increase in actin-myosin filament attachment to the cytoskeleton imposing increased internal loading with a concomitant slowing of the rate of ATP hydrolysis.

Efficiency Versus Power

Peak energy consumption of ASM attains a rate of ~0.12 nmol ATP mm^{-3} s^{-1} at peak power output. This is an approximate threefold increase from the ATP consumption rate during maximum isometric force (~0.04 nmol ATP mm^{-3} s^{-1}) [95]. In contrast, the rate of ATP hydrolysis in skeletal muscle increases from ~1.2 nmol ATP mm^{-3} s^{-1} during maximum isometric force generation to ~2.7 nmol ATP mm^{-3} s^{-1} during peak power output [95]. The energy cost of force generation in skeletal muscle ranges from ~16,000–35,000 nmol ATP s^{-1} mN^{-1} depending on fiber type. In contrast, the energy cost of force generation in smooth muscle is 6–14-fold lower at ~2,500 nmol ATP s^{-1} mN^{-1} [95]. The energy demands of skeletal and smooth muscle are reflections of the quality of their mechanical functions. Smooth muscles perform slower, yet sustained mechanical functions, while skeletal muscles are able to achieve more intense mechanical behaviors – the maximum power output of skeletal muscle is ~10–100 times that produced by smooth muscle [95]. Also, in skeletal muscle, there is a relatively large pool of phosphocreatine (PCr) that is available to buffer ATP hydrolysis transients [77]. In contrast, smooth muscle contains a relatively small PCr pool, which indicates a strong dependence on ATP production rather than on PCr buffering [56, 62, 100]. In summary, it appears that the skeletal muscle contractile response is optimized for maximum power output, while it could be argued that smooth muscle is optimized for maximum energy efficiency. Therefore, efficient matching of energy supply with energy demand may be a more significant governing factor for the ASM contractile response.

A Conceptual Framework for the Role of $[Ca^{2+}]_{mito}$ in Excitation-Energy Coupling in ASM

The mitochondria provide for most of the energy demands of the cell by replenishing ATP levels through oxidative phosphorylation. The overall outcome of oxidative phosphorylation is to consume both intramitochondrial NADH and O_2 to synthesize ATP from ADP and P_i. In vascular smooth muscle, it is observed that isometric force is strongly correlated to O_2 consumption [56, 62, 77], which implies a dependence on mitochondrial oxidative phosphorylation to supply ATP during contraction. Accordingly, this conceptual framework will define energy homeostasis of the ASM during contraction as a two-part system: ATP hydrolysis by actomyosin ATPase and ATP synthesis by oxidative phosphorylation.

Mitochondrial Ca^{2+} and ATP Production

Due to a substantial amount of evidence to support the role of Ca^{2+} in cellular energetics, this conceptual framework highlights intramitochondrial Ca^{2+}($[Ca^{2+}]_{mito}$) as principal effector of mitochondrial ATP production, although other effectors (such as ADP [16, 17], P_i [7], O_2, and fuel/substrate) likely play a role [11].

An emerging consensus is that elevated $[Ca^{2+}]_{mito}$ stimulates mitochondrial NADH production which in turn may stimulate ATP production (Fig. 5.4a) [25, 26, 36, 37, 104]. Increased NADH production in response to $[Ca^{2+}]_{mito}$ can be attributed to Ca^{2+}-sensitive intramitochondrial dehydrogenases associated with the tricarboxylic acid (TCA) cycle – pyruvate dehydrogenase (PDH), NAD-isocitrate dehydrogenase (ICDH), and oxoglutarate dehydrogenase (OGDH) – which are stimulated by different physiological levels of Ca^{2+} [25]. The mechanisms by which Ca^{2+} activates the dehydrogenases are not the same. Specifically, PDH is regulated by PDH-associated kinase and phosphatase – the phosphatase is Ca^{2+}-sensitive which promotes the active form of PDH. In the case of ICDH and OGDH, Ca^{2+} binds to the enzyme which increases the enzyme's affinity for substrate [25]. The relationship between dehydrogenase activity and Ca^{2+} can also be inhibited by an excess of end product (e.g., excess acetyl-CoA in the case of PDH) and can also be modulated by ATP/ADP ratios and substrate availability within the mitochondria [25]. In addition to $[Ca^{2+}]_{mito}$, increased $[Ca^{2+}]_{cyt}$ can also increase NADH levels in the mitochondria by stimulating mitochondrial shuttle systems (the glycerol phosphate shuttle [25] and the aspartate/glutamate transporters [75]).

The impact of increased NADH is summarized briefly (Fig. 5.4b). NADH donates electrons to the electron transport chain (ETC) within the inner membrane of the mitochondria. As the ETC catalyzes electron flow, H^+ is pumped against its concentration gradient out of the mitochondrial matrix. The final stage of the ETC requires O_2 to accept electrons (at Complex IV). Therefore, electrons flowing through the ETC results in O_2 consumption and, due to H^+ pumping, the formation

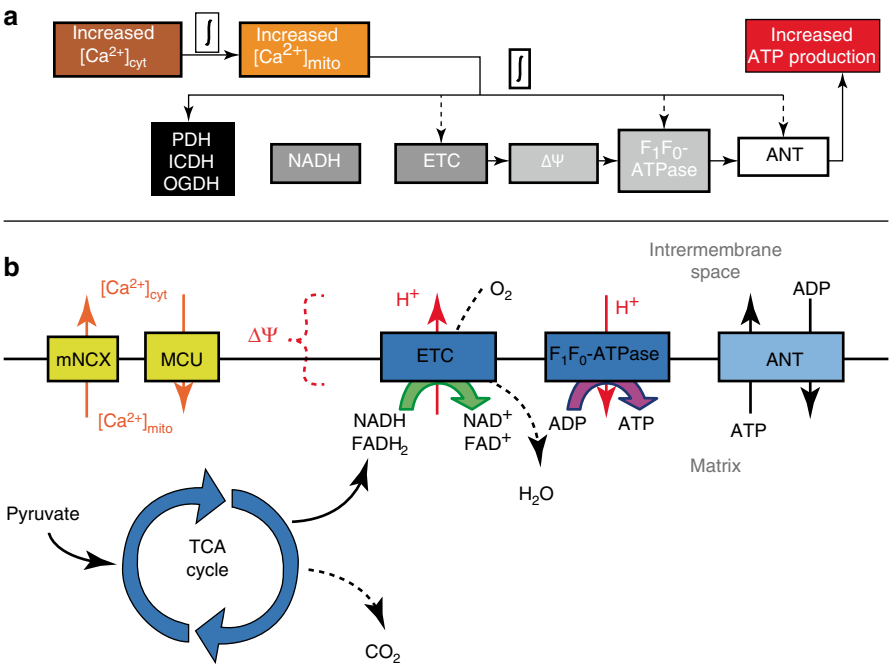

Fig. 5.4 (**a**) Excitation-energy coupling in airway smooth muscle cells indicating main temporal delays. The delay between $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mito}$ is estimated to be ~10 s while the delay between $[Ca^{2+}]_{mito}$ and increased ATP production is anticipated to be much faster. (**b**) ATP production in mitochondria summarized by main processes and intermediates also including the linkage between $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mito}$ as mediated by Ca^{2+} uptake by the MCU and Ca^{2+} release by the mNCX. *PDH* pyruvate dehydrogenase, *ICDH* NAD-isocitrate dehydrogenase, *OGDH* oxoglutarate dehydrogenase, *ETC* electron transport chain, $\Delta\Psi$ mitochondrial membrane potential, *ANT* adenine nucleotide transporter, *mNCX* mitochondrial Na^+/Ca^{2+} exchanger, *MCU* mitochondrial Ca^{2+} uniporter, *TCA cycle* tricarboxylic acid cycle

of a proton-motive force (pmf) which has electrical ($\Delta\Psi$) and chemical (ΔpH) features. The pmf favors the flow of protons into the mitochondrial matrix. The F_1F_o-ATPase (also called Complex V), which is permeable to H^+, uses the energy dissipated by pmf-driven proton flow into the matrix to synthesize ATP from ADP and Pi. Synthesized ATP is then transported to the cytosol in exchange for cytosolic ADP by the adenine nucleotide transporter (ANT).

Clearly, mitochondrial ATP production is a multistep process and, therefore, contains many potential regulatory sites upstream and downstream [11, 38, 90]. A recent review [36] outlines a role for Ca^{2+} to stimulate ATP production that is not solely dependent on NADH increases, but rather affects multiple sites to result in a "balanced activation" of mitochondrial energy production. For example, the impact of $[Ca^{2+}]_{mito}$ on mitochondrial ATP production is suspected to extend beyond dehydrogenase activation and may involve constituents of the ETC, F_1F_o-ATPase, substrate import, as well as others (Fig. 5.4a) [36].

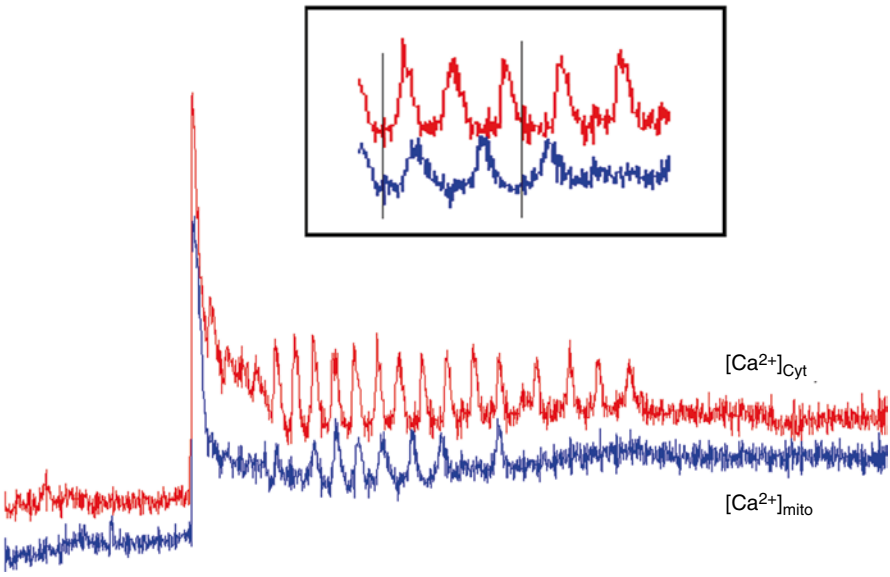

Fig. 5.5 In cells loaded with both fluo-3 (cytosolic Ca^{2+}, $[Ca^{2+}]_{cyt}$, red) and rhod-2 (mitochondrial Ca^{2+}; $[Ca^{2+}]_{mito}$, blue), 1 mM ACh-induced $[Ca^{2+}]_{cyt}$ oscillations that were reflected by delayed (~10 s), dampened $[Ca^{2+}]_{mito}$ oscillations. These data suggest that mitochondria take up $[Ca^{2+}]_{cyt}$ during agonist stimulation

Temporal Linkage Between Cytosolic [Ca^{2+}] and Mitochondrial [Ca^{2+}]

It has been found in many cell types [30, 46, 88], including smooth muscle [18, 24, 27, 70, 102], that an increase in $[Ca^{2+}]_{cyt}$ is temporally related to an increase in $[Ca^{2+}]_{mito}$ (Fig. 5.5). Accordingly, an increase in $[Ca^{2+}]_{cyt}$ is a main step in a multistep cascade, called excitation-contraction coupling (ECC), which causes muscle to generate force or contract in response to agonist stimulation. Given that $[Ca^{2+}]_{mito}$ can influence mitochondrial ATP production, the spatial and temporal aspects of $[Ca^{2+}]_{cyt}$ transients, which likely influence $[Ca^{2+}]_{mito}$, are anticipated to play an important role in excitation-energy coupling.

Mitochondrial Ca^{2+} Sensing and Decoding

Mitochondria are enclosed by two layers of membrane – the outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM). The region between the OMM and the IMM, called the intermembrane space, is commonly assumed to

be equivalent to the cytosol based on the assumption that the OMM is permeable. However, recent studies suggest that Ca^{2+} influx across the OMM is regulated via the voltage-dependent anion channel (VDAC or mitochondrial porin) [52, 82]. The IMM, which forms a pattern of folds, known as cristae, encloses the core of the mitochondria, the mitochondrial matrix. The IMM, which maintains a mitochondrial membrane potential ($\Delta\Psi$) due to oxidative phosphorylation, is impermeable to Ca^{2+}. IMM Ca^{2+} channels include the mitochondrial uniporter (MCU) which is largely responsible for Ca^{2+} uptake and the mitochondrial Na^{+}/Ca^{2+} exchanger (mNCX) which is largely responsible for Ca^{2+} release.

The electrochemical force that drives Ca^{2+} uptake through the MCU includes $\Delta\Psi$ and also the Ca^{2+} gradient across the IMM. Maximal activation of MCU in isolated mitochondria has been characterized to require Ca^{2+} levels that are much higher (>10 μM Ca^{2+}) than levels normally encountered in the bulk cytosol during stimulation (typically <2 μM Ca^{2+}) [43]. Therefore, it is expected that mitochondrial Ca^{2+} influx is prevented until a threshold of $[Ca^{2+}]_{cyt}$ is reached. The apparent inconsistency between the high threshold for MCU activation (>10 μM Ca^{2+}) and the $[Ca^{2+}]_{mito}$ increases despite subthreshold (<10 μM Ca^{2+}) $[Ca^{2+}]_{cyt}$ levels is reconciled by the "hotspot hypothesis" [85]. According to this hypothesis, the cytosol in the immediate vicinity of an open Ca^{2+} channel can transiently experience intense increases in Ca^{2+} (much higher than the bulk cytosol), called a "Ca^{2+} hotspot." Close proximity of mitochondria to open Ca^{2+} channels (within at least 100 nm) will expose the MCU to a microdomain of high Ca^{2+} levels (Ca^{2+} hotspot) potentially sufficient for activation [78, 84, 85].

Mitochondria also release Ca^{2+} via the mNCX which, like uptake via the MCU, may also participate in $[Ca^{2+}]_{cyt}$ regulation. The time-dependent behavior of $[Ca^{2+}]_{mito}$ can be modeled as a kinetic balance between Ca^{2+} fluxes through MCU versus mNCX. Conversely, mitochondrial Ca^{2+} uptake and release may participate in the regulation of $[Ca^{2+}]_{cyt}$ during agonist stimulation. For instance, compartmentalization of mitochondria with Ca^{2+} channels (PM or SR) could in turn affect the kinetics of those channels by regulating local levels of Ca^{2+} [14, 28, 45, 66, 67, 85, 87]. Mitochondrial production of ATP is accompanied by the generation of reactive oxygen species (ROS) which may also impact local Ca^{2+} channels [14, 20]. In addition to mitochondrial Ca^{2+} channels, there is also support for involvement of intramitochondrial Ca^{2+} buffering mechanisms, such as the formation of Ca^{2+}-P_i complexes, which may impact mitochondrial Ca^{2+} kinetics by limiting the activity of the mNCX over a range of high $[Ca^{2+}]_{mito}$ values [71].

Another mitochondrial Ca^{2+} uptake mechanism is the rapid uptake mode (RaM) [99]. RaM allows rapid Ca^{2+} influx but also rapidly becomes inactivated to permit brief bursts of Ca^{2+} uptake during the initial phase of a $[Ca^{2+}]_{cyt}$ transient [42, 99]. Since mitochondrial Ca^{2+} transport by RaM is activated at a lower $[Ca^{2+}]_{cyt}$ threshold [99], microdomain formation is not necessarily a strict requirement for mitochondrial Ca^{2+} sensing [43]. In addition to MCU, mNCX, and RaM, there are other types of Ca^{2+} flux pathways that have been described in the mitochondria, but the characterization of these alternate mechanisms is still in its infancy [78].

The decoding of a $[Ca^{2+}]_{cyt}$ signal by the mitochondria, in terms of ATP production, has been suggested by a number of investigators [37, 104]. In isolated mitochondria, where ATP production was measured by O_2 consumption, an increase in $[Ca^{2+}]_{mito}$ can increase ATP production rapidly (~200 ms or less) while taking seconds to achieve the maximum response [105]. In hepatocytes, IP_3-inducing hormone produced coupled $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mito}$ oscillations as well as NADH transients, which closely match the rising phase of increasing $[Ca^{2+}]_{mito}$ but decayed at a slower rate [46]. In HeLa cells, agonist-induced increases in $[Ca^{2+}]_{mito}$ were accompanied by increases in mitochondrial ATP production, measured by mitochondrial-targeted luciferase, which outlasted the duration of the Ca^{2+} signals [59].

Linkage of $[Ca^{2+}]_{mito}$ with $[Ca^{2+}]_{cyt}$ is dependent on the mode of $[Ca^{2+}]_{cyt\text{-}elevating}$ stimulus where IP_3-induced [46, 83, 84] or RyR [103] stimulus is the most effective. In contrast, $[Ca^{2+}]_{cyt}$ increases induced by thapsigargin (SERCA inhibition) are not effectively transmitted to mitochondria [46, 74].

Spatial Aspects of Mitochondrial Ca^{2+} Sensing

Mitochondria are spatially dynamic due to the ability to change shape and move within the cytosol. Mitochondrial shape changes can arise from the ability to fuse or divide among the mitochondrial population which may result in a spectrum of possible ultrastructures or shapes – from individual punctate structures to continuous, tubular network. The balance of fission versus fusion events is regulated by a set of dynamin-related GTPases: Drp-1, Mfn1/2, and OPA1. For more details, see reviews [12, 15]. The impact of fission/fusion of mitochondria likely also affects mitochondrial function in addition to structure. For example, overexpression of a pro-fission regulator, Drp-1, induced mitochondrial fragmentation and impaired the ability of a portion of the mitochondrial population to accumulate Ca^{2+} during agonist stimulation [101]. In general, it has been observed that changes in mitochondrial shape usually accompany changes in ATP production and vice versa; however, a unique relationship between fission-/fusion-controlled mitochondrial shape and ATP production is not defined [4, 33].

In addition to fission and fusion, mitochondria may also swell or change in volume related to ion transport. In liver cells, it is hypothesized that increases in $[Ca^{2+}]_{mito}$ can increase mitochondrial volume to stimulate ATP production [47, 48].

Additionally, the mitochondrial population can increase in mass in a process called biogenesis. Biogenesis is regulated by a number of factors, most notably peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), which is hypothesized to be stimulated by increased levels of $[Ca^{2+}]_{cyt}$. Increased mitochondrial biogenesis is implicated to be a factor involved in the pathophysiology of asthma and notably ASM cell proliferation and airway remodeling [106].

As in other cell types, mitochondrial location is dynamic in ASM cells (Fig. 5.6). Mitochondrial movement is mediated by interactions with the cytoskeleton and molecular motors. In brief, rapid and long-range movements require microtubule

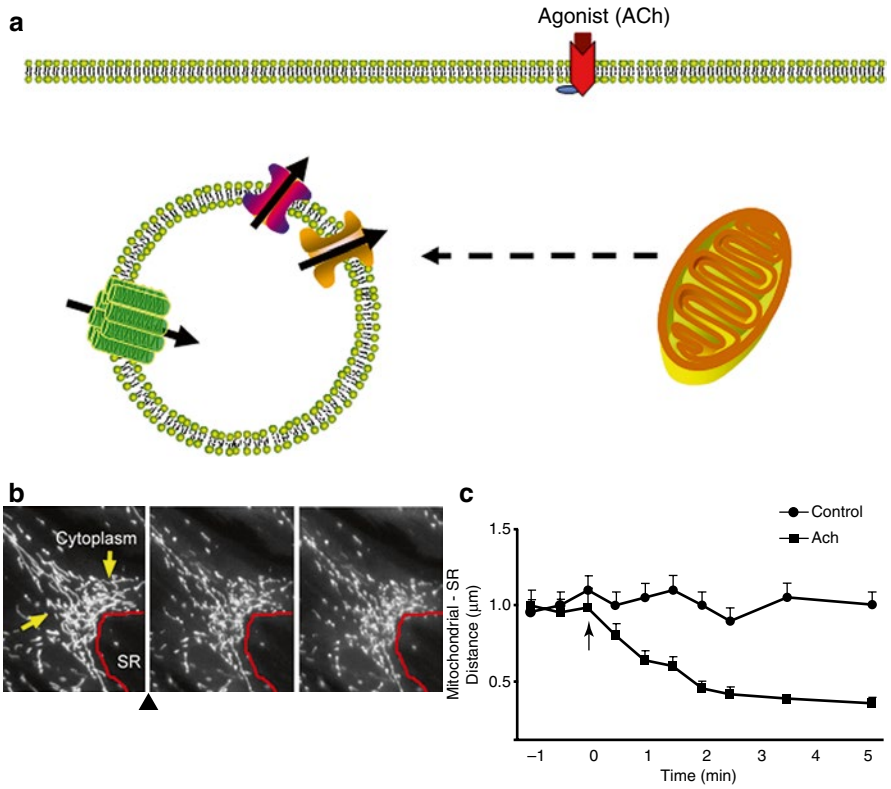

Fig. 5.6 (**a**) Conceptual model of mitochondrial movement during agonist stimulation. (**b**) ASM cells were loaded with 1 **μ**M MitoTracker Red and mitochondrial movement (*arrows*) towards SR (*red delineation*) measured using real-time confocal microscopy. (**c**) Upon exposure to ACh, mitochondria moved towards SR. This movement was completely inhibited by phalloidin (actin inhibitor, not shown), suggesting a role for actin filaments in mitochondrial movement

tracks and microtubule-associated motors, such as kinesin and dynein. F-actin and intermediate filaments are also involved, yet their role is under investigation [2, 6, 111]. Mitochondrial movement plays an important role in fission/fusion events and is likely to participate in sensing energy requirements [22, 107].

Mitochondrial movements can range from directed, long-range maneuvers to various wiggling-like motions [5] – mitochondrial movements can even be halted for a time period [51] which suggests that interaction of mitochondria with the cytoskeleton can be static reflecting a docking or stationary phase distinct from the motility phase. Mitochondrial movement is believed to be strongly correlated to $[Ca^{2+}]_{cyt}$ levels. In general, the pattern of mitochondrial movement followed the spatial and temporal pattern of $[Ca^{2+}]_{cyt}$ response which resulted in a redistribution of mitochondria to areas of high $[Ca^{2+}]_{cyt}$ [111]. Mitochondrial movement is also inhibited by depleting local ATP levels, which suggests that $[Ca^{2+}]_{cyt}$ and ATP may coordinate recruitment of mitochondria [10]. The stable positioning of

mitochondria near the SR can be physically reinforced by molecular tethers [34]. There is evidence that pro-fusion GTPase, Mfn2, is involved is such tethering [21]. Preliminary studies in our lab have indicated that stimulation of ASM cells with 10 μM histamine decreases wiggle-type mitochondrial motility – the decrease in this type of motility is more pronounced in the perinuclear region versus distal regions. However, longer-range, directed movements (observed in the distal regions only) increased following histamine stimulation. In nerve cells, prevention of actin polymerization, by exposure to latrunculin B, prevented mitochondrial docking into previously observed docking regions [13]. In ASM, a dynamic cytoskeletal structure may accommodate the effective matching of energy supply with demand by facilitating the docking of mitochondria into near-SR compartments (Fig. 5.6).

Since collective mitochondrial movement will affect mitochondrial distribution, many investigators have hypothesized that mitochondria strategically situate near regions of high ATP demand or regions with the potential for Ca^{2+} signal sensing and regulation – such as the SR (or ER for nonmuscle cells), plasma membrane, or the nucleus [10, 24, 30, 76, 111]. Following this hypothesis, a mitochondrial population is conceptualized to operate in heterogeneous subpopulations according to morphology and intracellular location. For example, the close proximity of a subpopulation of mitochondria to the SR likely allows maximal transmission of Ca^{2+} to those mitochondria and, as a result, increased ATP production. In fact, a significant portion of the mitochondrial population has been spotted in close proximity to the SR in ASM [19, 24] (Fig. 5.6) and other cells [74, 86, 92].

A mitochondrial subpopulation that is very close to the SR (which is dynamically recruited from the total population) is also likely employed in excitation-energy coupling; however, the influence of other factors in the context of ASM cells, including heterogeneity of the $[Ca^{2+}]_{cyt}$ response or cytoskeleton remodeling associated with ASM contraction (Fig. 5.6), pose interesting questions for further study.

Summary and Conclusions

The ASM cell is a physiological model of energy efficiency, especially in comparison to skeletal muscle. In particular, the dynamic nature of the ASM cell allow for adaptation to various stimuli while maintaining a stable time-course of muscle force. In fact, adaptation within the ECC process often improves efficiency by reducing energy cost, likely due to the dynamic remodeling of the cytoskeleton. Furthermore, given evidence that a change in metabolite levels is not detectable, credit must also be given to the processes that supply energy in just the right amount at the right time – excitation-energy coupling. The main feature of this process includes a linkage between an elevation of $[Ca^{2+}]_{cyt}$. and $[Ca^{2+}]_{mito}$ with subsequent decoding of $[Ca^{2+}]_{mito}$ into increased ATP production (Fig. 5.7).

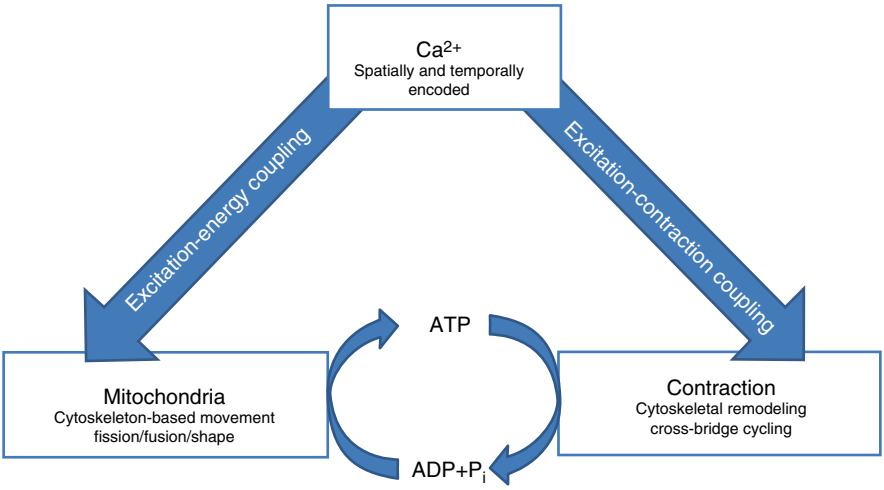

Fig. 5.7 The process of excitation-contraction coupling is proposed to be paralleled by an energetic response of the mitochondria, referred to here as excitation-energy coupling. Both processes are initiated by elevated $[Ca^{2+}]_{cyt}$ levels and result in stable cytosolic ATP levels during a contractile response. In ASM, these relationships may be influenced by spatial and temporal heterogeneity of the $[Ca^{2+}]_{cyt}$ response, contraction-associated cytoskeleton remodeling and crossbridge cycling, and finally cytoskeleton-based mitochondrial motility and fission/fusion events

The control of mitochondrial energetics is, at this stage, speculative. However, a few key observations support the conceptual framework that mitochondria are performing excitation-energy coupling. First of all, cytosolic metabolite levels do not significantly change, even during normal levels of energy consumption. Second, Ca^{2+} can signal contraction (ATP hydrolysis) and also significantly upregulate mitochondrial ATP production. Third, $[Ca^{2+}]_{mito}$ follows $[Ca^{2+}]_{cyt}$ during stimulation. Fourth, mitochondrial morphology and distribution determine the quality of mitochondrial Ca^{2+} sensing, and therefore there is likely also a connection between dynamic mitochondrial compartmentalization and efficient ATP production. In conclusion, ASM is an ideal experimental model to further explore the concept of mitochondrial excitation-energy coupling as a means of energy homeostasis.

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Chapter 6
Mitochondrial Lipid Peroxidation in Lung Damage and Disease

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Abstract Eukaryotic cells possess distinct and double-membrane encapsulated organelles, the mitochondria. The mitochondrion is the powerhouse of the cell responsible for energy production through oxidative phosphorylation. A dark side

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of the mitochondrion is its ability to generate reactive oxygen species (ROS) at specific sites of electron transport chain (ETC) arising from incomplete reduction of molecular oxygen. The mitochondrion is not only a source of toxic ROS but also their target and thus becomes vulnerable to oxidative attack. The ROS-mediated peroxidation of polyunsaturated fatty acids in mitochondrial membrane lipids leads to cell damage and injury of the tissue leading to pathophysiological states. The mitochondrial inner membrane that houses the ETC responsible for the cellular bioenergetics possesses a unique phospholipid, cardiolipin (CL), which is rich in polyunsaturated fatty acids susceptible to the ROS-induced peroxidation. Peroxidized CL has emerged as an important player in the mitochondria-driven oxidant lung injury, apoptotic cell death, and lung diseases. This review discusses the nature of the mitochondrial membrane lipids, mechanisms and consequences of the ROS-induced mitochondrial lipid peroxidation, and lipoperoxidative mechanisms of lung injury and diseases. Finally the pharmacological interventions of ROS-induced lung mitochondrial lipid peroxidation and mitochondriopathy that is involved in oxidant-induced lung damage and respiratory and lung diseases are also discussed.

Keywords Mitochondria • Lipid peroxidation • Cardiolipin peroxides • Pulmonary toxicity • Respiratory and lung diseases

Introduction

Eukaryotic cells have specialized membrane-bound autonomous organelles called "mitochondria" which are established as the "powerhouse of the cell" for the cellular energy production. In 1898, Carl Benda first named the microscopic granular threads the "mitochondria" (in Greek, *mitos* means "thread" and *chondrus* means "granule") [1]. The mitochondrion is considered as a cell within the eukaryotic cell (an endosymbiont) which has a speculative origin of protobacteria into archaea. The mitochondria utilize the energy substrates (e.g., glucose) in the presence of oxygen and generate energy (adenosine triphosphate, ATP) through oxidative phosphorylation. Other than just being the powerhouse of the cell, the mitochondria have emerged as dynamic organelles of the cell, which regulate diverse and critical cellular functions including metabolic events, cellular calcium homeostasis, cell signaling, cell cycle, vesicle trafficking, development, redox status, apoptosis, autophagy, and cellular death [2–6]. The mitochondrion is a unique organelle with two membranes encircling the organelle, the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM). The space between the outer and inner membranes is called the mitochondrial intermembrane region [6]. Although the outer membrane houses channels and operates intramitochondrial transport of solutes, the membrane acts as a selective barrier between the mitochondria and cytosol for the transport of mitochondrial proteins. The IMM is extensively folded to form the cristae which act as the site for oxidative phosphorylation and production of the energy currency, the high energy phosphate, 5′-adenosine triphosphate

(ATP). The mitochondrial inner membrane encapsulates a metabolically active area called "matrix" that is rich in enzymes which catalyze the citric acid cycle, oxidation of fatty acids, and assembly of the mitochondrial genome [7]. As an autonomous eukaryotic intracellular double-membrane-bound organelle, the mitochondrion contains its own DNA, RNA, and protein synthesis machinery and is capable of the mitochondrial biogenesis [8]. Although the mitochondrion is an autonomous organelle, it is under the transcriptional control of both nuclear and mitochondrial genes. The mitochondrial genome encodes 37 genes among which 24 genes are responsible for encoding the 13 crucial enzymes that conduct the oxidative phosphorylation and ATP generation [5, 6]. The OMM and IMM are bilayer membranes with specific lipid and protein compositions that dictate the structure and function of the unique eukaryotic organelle, the mitochondrion.

Mitochondria as the Source of Reactive Oxygen Species and Mediator of Oxidative Stress

Molecular oxygen (O_2) acts as a substrate for energy (ATP) generation from the energy substrate through oxidative phosphorylation. In the cristae of the inner mitochondrial membrane, the electron transport chain (ETC) converts O_2 to water (H_2O) by 4-electron reduction while generating ATP through the generation of transmembrane electrochemical gradients [1, 9–11]. Leakage of electrons during uncoupling of the oxidative phosphorylation in ETC leads to the generation of oxygen radicals (e.g., superoxide anion, O_2^-) at complexes I and III in the mitochondrial matrix [12]. The mitochondrial superoxide dismutase (Mn-SOD or SOD-2) dismutates O_2^- to hydrogen peroxide (H_2O_2) which further leads to formation of the hydroxyl radical (OH) in the presence of iron (Fe^{2+}). H_2O_2 generated by the mitochondria can also undergo efflux from the organelles and reach the cellular cytosolic compartment [12]. Collectively, O_2^-, H_2O_2, and OH are called "reactive oxygen species" (ROS) [13]. ROS, being highly reactive in nature, attack membrane lipids, proteins, and DNA and oxidatively modify them leading to the alterations of the mitochondrial structure and function. Thus, the mitochondria-generated ROS have been established to cause oxidative stress in the mitochondria and cells [9, 10, 14]. The mitochondria have also been shown to contain nitric oxide synthase (mtNOS) that generates NO [15, 16]. The mtNOS-generated NO reacts with O_2^- to form highly reactive and toxic peroxynitrite species that are known to cause oxidative and nitrative stress in the mitochondria and cell [15]. The mtNOS-generated reactive nitrogen species (RNS) are also known to modulate cellular signaling, alter cellular redox status, regulate cell proliferation, and activate proapoptotic pathways [17]. Glutathione (GSH) is the major regulator of thiol-redox status and GSH-dependent antioxidant enzymes, and mitochondria are the chief regulators of cellular GSH homeostasis [18]. Perturbations in the mitochondrial GSH status will alter the mitochondrial and cellular redox homeostasis leading to cellular distress [19]. The mitochondria also possess tightly regulated enzymatic antioxidant machinery

that consists of but is not limited to Mn-SOD, glutathione peroxidase (GSH-Px), thioredoxin, and peroxiredoxin [20] in addition to the nonenzymatic antioxidants such as coenzyme Q_{10} and vitamin E. The function of these enzymatic and nonenzymatic antioxidants is to scavenge ROS and RNS and offer protection against oxidative stress in the mitochondria.

Mitochondrial Membrane Lipid Organization

Cellular membranes including the plasma membrane are made up of phospholipids (PLs), sterols, sphingolipids, and proteins. Biological membranes are not merely structural entities providing selective barrier and structural properties to cells but also specialized entities that provide functional uniqueness to each and every intracellular compartment. Typically, the PLs which form the bilayer are the major backbone of the membrane. Although the mitochondria synthesize certain lipids independently, they acquire lipids made by the endoplasmic reticulum (ER). The dependence of mitochondrion on the ER for constant supply of lipids is crucial for the maintenance of structure and function of the organelle. Among the PLs present in the majority of the biological membranes of the eukaryotic cells analyzed so far, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the major PLs present in the plasma membrane and organelle membranes including those of the mitochondria [21]. The mitochondrial membranes contain PC and PE up to ~80 % of the total PL content followed by the third largest PL component, cardiolipin (CL) that occupies up to 10–15 % of the total mitochondrial membrane PL pool. Other PLs including phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid (PA), sphingolipids, and sterols constitute a small but significant mitochondrial membrane PL pool [21]. Cholesterol has been reported to be present as a minor lipid component in the OMM of mammalian cells [21]. Nonetheless, lipid composition of the mitochondria in different species known so far appears to be similar with the exceptions of the plant mitochondria and microorganisms which lack sterols and sphingolipids [21]. Studies have revealed that the OMM and IMM have distinct PL composition typical of each membrane. More noticeably, the IMM contains more CL as compared to that of the OMM [21]. The site of CL remodeling is the IMM, and unlike some mitochondrial PLs which are synthesized extramitochondrially such as the ER and then incorporated into the mitochondrial membranes upon transfer from the source, CL is tightly associated with the site of its synthesis [22]. Above all, the lipids are asymmetrically distributed in the mitochondrial membranes. In the rat liver mitochondria, PE has been shown to be distributed up to 77 % in the OMM outer bilayer oriented towards cytoplasm where PC nearly has been shown to be equally present in two sides of the OMM bilayer [21]. Thus, the differences in composition and arrangement of different lipid species in the OMM and IMM contribute to their typical shape and structure which dictate their functions [23].

Among the PLs of the mitochondrial membranes, CL of the IMM which comprises 18 % of the total membrane lipid pool stands out as an important PL species

for the mitochondrial functions including energy generation through oxidative phosphorylation, apoptotic cell death, orientation, organization, and function of proteins in the mitochondrial membranes [1, 21, 24, 25]. CL, also called "diphosphatidylglycerol" or "bisphosphatidyl glycerol lipid" is a unique mitochondria-specific PL and can be considered as the fingerprint or signature of the mitochondria in the eukaryotic cells [22, 26, 27]. The chemical structure of CL is 1′,3′-bis(1,2-diacylglycero-3-phospho)glycerol with four fatty acyl chains (tetra-acyl) esterified to the hydroxyl groups in a stereochemical fashion [26]. Although 625 different molecular species with five different fatty acyl chains and 38,416 molecular species with 14 different fatty acyl chains in the yeast and human mitochondria, respectively may be possible, the occurrence of real number of CL molecular species containing different fatty acyl chains is considerably lesser due to the selection of only two types of fatty acids for esterification in CL [26]. Thus, CL is highly asymmetrical and the two phosphorus groups offer negative charge to the lipid at physiological pH [27]. The mammalian mitochondrial CL up to 80 % of the entire CL pool has been shown to contain predominantly linoleic acid (C18:2), and the rest of the 20 % of CL has been observed to contain other fatty acids including oleic acid (C18:1) and linolenic acid (C18:3) [24]. CL is a crucial mitochondrial IMM PL that is vital for the mitochondrial functions including respiration, ATP synthase function, cytochrome *c* oxidase activity in complex IV, functions of different transporters, anchoring of cytochrome *c* to IMM, proton transfer through cytochrome bc_1, maintenance of the IMM fluidity and osmotic stability, mitochondrial biogenesis, cytochrome *c* release, and apoptotic cell death [28]. However, under certain pathophysiological conditions and aging, the fatty acid composition of CL has been shown to change due to alterations in the genes responsible for CL metabolism leading to CL remodeling that is responsible for the altered mitochondrial functions [27–29].

Lipid Peroxidation in Mitochondria

Polyunsaturated fatty acids (PUFAs) of the membrane PLs are vulnerable to the free radical-mediated lipid peroxidation through enzymatic and/or nonenzymatic mechanisms and further generate radicals. The mitochondria are known to possess the enzymatic machinery that generates ROS (at the ETC) and RNS (from mtNOS) that are capable of causing peroxidation of PUFAs in membrane PLs. ROS and RNS originating from the mitochondrial and extramitochondrial sources are capable of inducing the peroxidation of the membrane PL PUFAs, and the mitochondrial membranes are not an exception to this. Lipid peroxides formed during the peroxidation of membrane lipids undergo further decomposition aided by transition metals (e.g., Fe^{2+}) and form highly reactive lipid peroxy and alkoxy radicals which will further continue the lipoperoxidative chain reaction and cause oxidative deterioration of the membranes [30, 31]. PUFA peroxides, which are classified among ROS [32], also form highly reactive carbonyls (e.g., 4-hydroxy-2-nonenal, 4-HNE) which are strong electrophiles reacting with the –SH groups of GSH and thus cause alterations

in the redox status, structure and function of several proteins and enzymes, cellular signaling cascades, and gene expression at the mitochondrial and cellular levels. Acrolein, a highly reactive and toxic carbonyl present in the tobacco smoke and polluted air, has been identified to originate from the peroxidation of PUFAs that causes cellular distress [33]. Reactive carbonyls of lipid peroxidation origin have been also shown to react with the nucleic acids (RNA and DNA) and alter the structure and function of the genome resulting in altered protein synthesis, gene expression, and mutations. The membrane PLs having either PUFA hydroperoxide moieties or reactive carbonyls not only cause the membrane damage and dysfunction but also contribute to the oxidative stress in the mitochondria and cells. Both mitochondria and cells have been shown to possess specific enzymatic antioxidant defenses to protect against the membrane lipid peroxidation. Catalase, a heme enzyme that converts H_2O_2 to H_2O, is present in the cells mostly in the peroxisomes but not in the mitochondria. Glutathione peroxidase (GSH-Px or GPx), either selenium-dependent or selenium-independent, utilizes both H_2O_2 and non-esterified (free) PUFA hydroperoxides as substrates and converts them into water and hydroxy-PUFAs at the expense of GSH (reduced form) as a cofactor that is converted to glutathione disulfide (oxidized form, GSSG) [32]. GSSG is recycled and converted back to GSH by GSH reductase with the utilization of NADPH, the reducing cofactor. However, another enzyme specific to PL-esterified PUFA hydroperoxides, called the PL hydroperoxide GSH peroxidase (PHGPx), selectively acts on the PL-esterified PUFA hydroperoxides with the aid of GSH as a cofactor, converts them into the respective hydroxyl species, and thus protects against the membrane PL peroxidation. Other thiol-redox-stabilizing enzymes including the peroxiredoxin (Prx) also detoxify H_2O_2 by converting it into H_2O through the oxidation of thioredoxin (TRx), and reduced TRx is regenerated back by TRX reductase at the expense of NADPH as the cofactor [32]. The selenium-containing enzymes, GPx, PHGPx, and thioredoxin reductase have also been shown to be present in the mitochondria which tightly regulate the thiol-redox homeostasis in the organelle protecting both mitochondria and the cell from oxidative stress [34–36].

CL, the unique and important PL of the mitochondria, is extremely vulnerable to peroxidative attack by ROS by virtue of its richness in PUFA, predominantly linoleic acid (C18:2) up to >80 % [37, 38]. Cytochrome *c* has been shown to catalyze the peroxidation of CL that leads to the execution of apoptosis in HeLa cells [39]. Although CL can undergo peroxidation through conventional means of free radical attack, cytochrome *c* catalyzes peroxidation of CL through its peroxidase activity, utilizing CL as a substrate in a cytochrome *c*-CL complex, which is crucial for apoptosis as observed in the HL-60 cells [40]. In this study, the authors have also demonstrated the oxidant-induced peroxidation of membrane PLs such as PC, PS, and CL in the cells and the cytochrome *c* + H_2O_2-catalyzed formation of monohydroperoxy and monohydroxy CL in the PC liposomes containing tetralinoleoyl CL (Fig. 6.1). On the other hand, other peroxidatively modified species of CL such as the carbonyl-containing PUFA (C18:2) of CL are also expected to arise during the peroxidation of CL. Furthermore, CL in PC liposomes has been shown to undergo peroxidation induced by air, singlet oxygen, and free radical initiator leading to the formation of

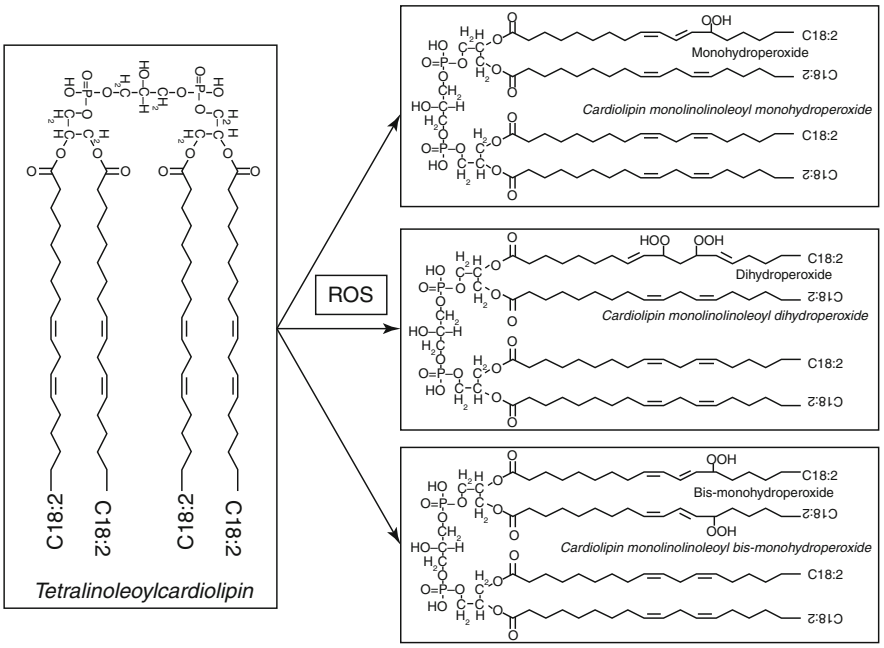

Fig. 6.1 ROS-mediated peroxidation of CL forming different molecular species of PUFA hydroperoxides. ROS causes peroxidation of linoleic acid in CL and forms monohydroperoxide and dihydroperoxides on one linoleate ester. Also, in some cases, ROS-induced oxidation of CL leads to the formation of bis-monohydroperoxides on two adjacent linoleate esters

monohydroperoxides and bis-monohydroperoxides of CL (Fig. 6.1) [41]. However, in this study, substantial quantity of dihydroperoxides of CL catalyzed only by singlet oxygen has been reported [41]. Thus, cytochrome *c* has been called "CL oxygenase" that is activated under apoptotic conditions leading to selective peroxidation of CL [42]. Disturbance of the tight association between CL and cytochrome *c* due to ROS attack and loss of CL content in the mitochondria is known to cause the release of cytochrome *c* from the mitochondria and triggering of apoptosis through a redox-mediated mechanism, assembly of apoptosomes, and activation of caspases [42, 43]. In this scenario, PS, the anionic PL of the plasma membrane, enters as a new player in the execution of apoptosis through the mitochondrial pathway. PS is also oxidized by cytochrome *c* released into cytosol by the mitochondria in the manner akin to the oxidation of CL by cytochrome *c*. Consequently, the oxidized PS of plasma membrane and the peroxidation of CL in the mitochondrial membranes contribute to the apoptosis [42]. Peroxidation of CL has been shown to exert dire effects on the functions of the mitochondria through elevated calcium (Ca^{2+}) levels that are associated with the mitochondria-mediated pathophysiological conditions and diseases [44]. On the other hand, deficiency of CL in the HeLa cell model has been shown to augment resistance to apoptosis through inhibiting the association of CL-cytochrome *c* complex and peroxidation of CL [45]. Abnormal increase of

cholesterol levels in the mitochondrial membranes has been shown to lower the mitochondrial GSH redox status leading to CL peroxidation through tumor necrosis factor (TNF)-induced ROS formation, which subsequently causes alterations in the mitochondrial membrane properties such as permeabilization [46]. Thus, the cholesterol-mediated CL peroxidation in the mitochondria appears to be a key regulator of cell death. Nevertheless, PHGPx, the mitochondrial antioxidant enzyme specific to PL-hydroperoxides, has been shown to lower the formation of CL-hydroperoxides in the mitochondria and act as antiapoptotic factor in the leukemia cells [47].

Environmental Metals, Particulate Matter (PM), Lipid Peroxidation, and Lung Mitochondria: Respiratory and Lung Diseases

The airborne trace heavy metals and particulate matter (PM) have been shown to pose a serious threat to the respiratory and lung health of humans through the generation of ROS and oxidative stress [48]. In this context, the role of mitochondria-generated ROS in the pathogenesis of human diseases has been emphasized [49]. Needless to mention, the ROS-induced membrane lipid peroxidation and mitochondrial dysfunction as the underlying mechanisms of tissue damage cannot be ruled out. The heavy metal cadmium (Cd) is an established environmental pollutant, etiological factor in emphysema, and a carcinogen that induces lung cancer [50]. In order to establish the Cd-induced damage of the lung, a study has been conducted which reveals that Cd causes damage of the MRC-5 fetal lung fibroblasts through ROS production, lipid peroxidation, and alterations in the mitochondrial membrane potential [50]. The results of this study confirm that the ROS-mediated lipid peroxidation and mitochondrial alterations are involved in the damage of lung fibroblasts as an underlying mechanism of lung toxicity of Cd. Atmospheric residual oil fly ash (ROFA) PM has been shown to be associated with many environmentally induced lung diseases such as the chronic obstructive pulmonary disease and lung cancer [51]. It has been shown that the ROFA PM causes cytotoxicity to the human alveolar epithelial cells (A549 cells) in vitro through the formation of ROS, DNA damage, lipid peroxidation, and mitochondrial alterations [51, 52]. Thus, the transition metals (vanadium, iron, and nickel) present in the ROFA PM have been identified as potential initiators of mitochondrial dysfunction in the alveolar epithelial cells through oxidative stress (ROS production and lipid peroxidation) that could be critical in the onset and progression of the environmental lung diseases.

The PM air pollution of the urban microcosms is a global health concern due to its association with the respiratory and lung diseases, cardiovascular diseases, morbidity, and mortality among adults as well as children as revealed by the epidemiological studies [53]. Especially, the respirable PM_{10} has been shown to be associated with the ROS production and oxidative stress, causing inflammation through the activation of critical signaling pathways, and therefore, PM_{10} is considered an environmental risk factor in the aggravation of respiratory and lung diseases [53]. Airborne PM such as silica, TiO_2, nanoparticles, and those generated by the

combustion of wood and fossil fuels have been shown to cause the intracellular formation of ROS resulting in oxidative stress, lipid peroxidation, DNA damage, depletion of antioxidants, and alterations of the mitochondrial functions in animal models and humans [54]. The urban airborne $PM_{2.5}$ collected from the Dunkerque City air in France has been shown to cause cytotoxicity, oxidative stress (lipid peroxidation), DNA damage, mitochondrial dysfunction, and inflammatory response in the human lung epithelial L132 cells in vitro [55]. The $PM_{2.5}$ has also been shown to cause apoptosis associated with tumor necrosis factor-α secretion, caspase activation, DNA damage, and mitochondrial involvement in the human lung epithelial L132 cells in culture [56]. These studies have revealed that the metals (especially Al, Fe, and Pb) and polycyclic aromatic hydrocarbons (PAHs) present in the urban airborne $PM_{2.5}$ could be involved in causing oxidative stress (lipid peroxidation) and associated mitochondrial alterations and apoptosis in the lung epithelial cell model [55, 56]. Another study conducted to establish the mechanisms of damage of the lung epithelial cells (A549 cells) induced by the airborne $PM_{2.5}$ of Abidjan, France, has demonstrated that the $PM_{2.5}$-induced oxidative damage of A549 cells in culture has been mediated by oxidative stress including the loss of antioxidant defenses, elevated lipid peroxidation, and alteration in the mitochondrial dehydrogenase activity [57]. Also, this study reveals that the Abidajan $PM_{2.5}$ contains trace metals that are capable of inducing oxidative stress through ROS generation, antioxidant depletion, oxidative stress including lipid peroxidation, and the mitochondrial alterations. Overall these studies suggest the role of ROS and oxidative stress, lipid peroxidation, mitochondrial dysfunction, and apoptosis in the trace metal- and airborne PM-induced cellular damage of the respiratory tract leading to the respiratory and pulmonary diseases including lung cancer [58].

Herbicides and Lipid Peroxidation and Lung Mitochondria: Respiratory and Lung Diseases

Paraquat, the extensively used bipyridium herbicide (1,1′-dimethyl-4,4′-bipyridium dichloride) in addition to damaging several organs, is known to cause oxidative injury to the lung leading to pulmonary fibrosis [59]. Paraquat has been shown to cause O_2^- formation through the activation of the mitochondrial NADH-ubiquinone oxidoreductase of the complex I and the production of paraquat radicals, which cause oxidative stress and lipid peroxidation in the lung submitochondrial particles of the paraquat-administered rats [60]. Furthermore, it has been shown that cytotoxicity of paraquat in the rat lung following in vivo administration of the herbicide has been mediated by the mitochondrial dysfunction through complex I activation and lipid peroxidation in the inner membrane [61]. More interestingly, the role of mitochondrial lipid peroxidation in mediating the paraquat-induced lung damage and protection of associated lung fibrosis by the heat-shock protein 60 (HSP60) in the paraquat-intoxicated rats has been observed [59]. Hence, lipid peroxidation of the IMM appears to play a crucial role in lung damage and fibrosis induced by paraquat through oxidant production and oxidative stress.

Carcinogens, Lung Mitochondria, and Lipid Peroxidation: Lung Cancer

Several toxicants including xenobiotic chemicals such as chemical carcinogens are known to cause lung cancer through the mitochondrial lipid peroxidation and oxidative stress [62–64]. The well-established carcinogen benzo[a]pyrene (B[a]P) has been shown to cause elevated extent of the mitochondrial lipid peroxidation along with the lowered antioxidant status and decreased activities of the mitochondrial bioenergetic enzymes in the neoplastic lung tissue of mice [62]. Capsaicin, a natural product occurring in hot peppers (*Capsicum annuum*) has been shown to protect against the B[a]P-induced lipid peroxidation and alterations in the lung mitochondria and exert chemopreventive action against lung cancer, suggesting that the mitochondrial lipid peroxidation has an apparent role in the chemical carcinogen-induced lung cancer in the mouse model. In another study, the carcinogen B[a]P has been shown to cause the mitochondrial damage in the mouse lung through the ROS formation, lipid peroxidation, loss of antioxidant status, and mitochondrial membrane damage and dysfunction, all of which have been shown to be protected by crocetin, a saffron (*Crocus sativus*) carotenoid [63]. Since B[a]P is a PAH carcinogen also present in cigarette smoke and known to cause lung cancer, a study has been conducted on the B[a]P-induced lung mitochondrial oxidative damage in vivo in mice and its protection by baicalein, a well-established flavonoid and lipoxygenase inhibitor present in the roots of *Scutellaria baicalensis* [64]. This study has demonstrated that B[a]P causes the mitochondrial lipid peroxidation and formation of DNA adducts with malondialdehyde (MDA), an end product of lipid peroxidation. Also, this study has revealed that baicalein exhibits chemotherapeutic action in protecting against B[a]P-induced lung cancer possibly through attenuation of the mitochondrial damage caused by lipid peroxidation and formation of MDA-DNA adducts. Another in vivo study in mice has shown that the tobacco carcinogen B[a]P induces lung cancer through oxidative stress, lipid peroxidation, perturbation of antioxidant status, alterations in the electron transport chain activities and energy (ATP) generation, and ultrastructural modifications in the lung mitochondria [65]. Interestingly, hesperidin, a flavanone present in citrus fruits, has been shown to offer protection against the B[a]P-induced lung mitochondrial damage including lipid peroxidation, suggesting the antineoplastic and mitochondrial protective actions of hesperidin in the carcinogen-induced lung cancer. Another in vivo investigation in mice has revealed that elevation of the lung mitochondrial lipid peroxidation and antioxidant depletion appear to play crucial roles in the B[a]P-induced lung cancer [66]. Furthermore, this study has demonstrated the protective actions of piperine (an alkaloid present in the black pepper, *Piper nigrum*) against the B[a]P-induced lung mitochondrial lipid peroxidation and antioxidant alterations and also against the B[a]P-induced lung cancer. Taken together, these studies highlighted above have demonstrated that the mitochondrial lipid peroxidation and dysfunction are tightly associated with the chemical carcinogen-induced lung cancer, and natural products of plant origin could act as antineoplastic agents in the lung through ameliorating the mitochondrial lipid peroxidation.

Hypoxia, Hyperoxia, Oxidants, and Radiation Modulate Lipid Peroxidation and Lung Mitochondrial Function

Disturbances in lung oxygen concentrations (pO_2) such as hypoxia and hyperoxia that generate ROS and exposure to environmental oxidants including nitrogen dioxide (NO_2) and ozone (O_3) have been shown to cause lung injury wherein the membrane lipid peroxidation and the mitochondrial damage appear as important players [67, 68]. Hyperoxic injury has been established to be mediated by generation of oxygen free radicals, lipid peroxidation, and oxidative stress [69]. Hyperoxic lung injury is not an exception to this mechanism of injury, and antioxidant defense systems are known to protect against the hyperoxia-induced lung damage [70]. GSH has been established as a critical player in maintaining the thiol-redox homeostasis and protection against oxidative injury in the lung [71]. Along those lines, it has been shown that the hyperoxic lung injury in mice in vivo associated with the increased mitochondrial ROS production and lipid peroxidation (8-isoprostane formation) is attenuated by the cellular thiol-redox enhancer/stabilizer, N-acetyl-L-cysteine (NAC), through enhancement of GSH levels and induction of Mn-superoxide dismutase (Mn-SOD) [72]. Thus, in this study NAC has been shown to protect against the hyperoxic lung injury through attenuation of the mitochondrial oxidative stress by enhancing the antioxidant status. Acute hypoxia has also been shown to cause ROS formation and elevation of extent of lipid peroxidation in the lung mitochondria of rats [68]. However, in this study, it has been demonstrated that daily exposure to moderate hypoxia (5 min exposure to 10 % O_2) alternatively with hyperoxia (5 min exposure to 30 % O_2) for 2 weeks lowers the hypoxia-mediated lipid peroxidation and elevates GSH levels leading to protection of the acute hypoxia-induced mitochondrial damage in the lung. Also, the results of this study suggest that exposure of the lung to alternate cycles of hypoxia and hyperoxia induces adaptive responses for the lung to cope with the oxidant damage during acute hypoxic stress through protection against oxidative stress (lipid peroxidation) in the lung mitochondria.

As discussed earlier, the distinct and intriguing feature of the mitochondrion is its possession of the unique phospholipid CL in the inner mitochondrial membrane which is highly enriched with PUFAs. CL has been unequivocally established as a master switch to initiate apoptosis [73]. Peroxidation of the mitochondrial CL, release of cytochrome *c*, and induction of apoptosis in several systems have been established and shown to play crucial roles in several human diseases [74–77]. Incidentally, it is rapidly emerging that the oxidant-mediated respiratory and lung diseases are either associated with or mechanistically regulated by the mitochondrial CL peroxidation and associated apoptotic cell death. Utilizing the high-performance liquid chromatography (HPLC) and mass spectrometry (MS) methods, it has been demonstrated that the mitochondrial CL undergoes peroxidation forming hydroperoxy and hydroxy species of PUFA esters and thus contributes to the induction of inflammation and apoptosis in the pulmonary artery endothelial cells and lung tissue under hyperoxic stress and following inhalation of carbon nanotubes [78]. With the use of oxidative lipidomics technology aided by the electrospray ionization MS

(ESI-MS), it has been elegantly shown that the hyperoxia-induced acute lung injury in mice in vivo is mediated by peroxidation of the lung mitochondrial CL and non-mitochondrial PS [79]. Furthermore, this study demonstrates that hyperoxia causes peroxidation of CL and PS in the mouse lung endothelial cells leading to apoptosis, which is significantly protected by the mitochondria-targeted free radical scavenger, hemi-gramicidin S conjugate with XJB-5-131(a nitroxide) [79]. Overall, these studies provide convincing evidences supporting the role of mitochondrial CL peroxidation in acute lung injury caused by hyperoxia.

Rats subjected to thoracic γ-ray irradiation have shown elevated lipid peroxidation in the lung mitochondria as the underlying mechanism for radiation-induced interstitial pneumonitis through oxidative stress [80]. This observation has also paralleled with the decreased activities of catalase and SOD in the lung cytosol, suggesting that the radiation-induced mitochondrial lipid peroxidation has been exacerbated by the loss of activities of antioxidant enzymes. The gaseous air pollutant and constituent of cigarette smoke nitric oxide (NO) and the product of its reaction with superoxide (O_2^-) peroxynitrite ($ONOO^-$) have been shown to induce oxidative stress and cell death in the lung epithelial cells [81]. Also, this study reveals that the NO-induced lung epithelial cell death is different from that induced by $ONOO^-$ as the former and latter being apoptotic and necrotic, respectively. Furthermore, this study suggests that the NO-induced apoptotic cell death of the lung epithelial cells could be due to the mitochondrial damage, cytochrome *c* release, and caspase activation. The $ONOO^-$-induced necrotic cell death could also be mediated by lipid peroxidation since $ONOO^-$ has been observed to induce cellular lipid peroxidation. Vitamin A supplemented at clinical doses as retinol palmitate (1,000–9,000 IU/kg bw/day for 28 days) has been shown to exert prooxidant action in exacerbating the NO-induced lung mitochondrial oxidative and nitrative stress through lipid peroxidation, protein carbonyl formation, and elevated levels of 3-nitrotyrosine formation [82]. Although the nitric oxide synthase (NOS)-specific inhibitor, L-NAME has been observed to offer marginal protection against the vitamin A-induced lung mitochondrial oxidative and nitrative stress, this study suggests that the exact mechanism of exacerbation of the NO-induced lung mitochondrial oxidative and nitrative damage by vitamin A remains elusive.

Tobacco Smoke, Mitochondria, and Lipid Peroxidation

Tobacco smoke including cigarette smoke is known to contain a plethora (>3,800) of toxic compounds that are capable of mediating the free radical-mediated reactions in the respiratory tract leading to lipid peroxidation, oxidative stress, damage of the macromolecules (proteins and DNA), and ultimately lung damage and pulmonary diseases such as emphysema and lung cancer [83, 84]. Therefore, it appears that the mitochondria of lung exposed to tobacco smoke are vulnerable to oxidant-induced damage that is likely to mediate the pathophysiological condition of the organ. The mitochondrial DNA (mtDNA) in humans has been shown to undergo mutations as a

result of aging and environmental changes, and the role of oxidative stress in causing mutations of the mtDNA is not ruled out [85]. It has been shown that significant base pair deletion mutations of the mtDNA in lungs of smokers and aging individuals are associated with lipid peroxidation and oxidative damage of DNA [85]. This study underscores the critical role of lipid peroxidation in augmenting oxidative DNA damage in lung tissue of smokers leading to mutations of the mtDNA. It is increasingly becoming evident that environmental factors exacerbate the tobacco smoke-induced respiratory diseases such as asthma through synergistic actions wherein the oxidative stress-mediated lung mitochondrial damage is a critical player [86]. The combined exposure of particulates in printer emissions and environmental tobacco smoke has been shown to cause oxidant (ROS) generation, lipid peroxidation, antioxidant depletion, and mitochondrial damage and dysfunction in the lungs of asthmatic mouse model [86]. Thus, this study has revealed that the environmental factors such as the printer particulate emissions exacerbate pulmonary diseases such as asthma through lipid peroxidation, oxidant damage, and lung mitochondrial damage and dysfunction. Exposure of cigarette smoke extract (CSE) has been shown to cause damage to the human alveolar epithelial cells in vitro [87]. This study has revealed that the CSE-induced alveolar epithelial cell injury (necrosis and apoptosis) are associated with lipid peroxidation and altered mitochondrial membrane potential suggesting that the lipoperoxidative mitochondrial damage and dysfunction in the alveolar epithelial cells appear to play important roles in the cigarette smoke-induced lung damage and diseases such as emphysema.

Lipid Peroxidation Products, Eicosanoids, and Lung Mitochondria

PUFAs of membrane lipids undergo free radical-mediated peroxidation in the living cells leading to the formation of diverse and highly reactive compounds including the fatty acid hydroperoxides, aldehydes, and electrophilic carbonyls which not only react with cellular macromolecules (proteins and nucleic acids) but also cause cellular dysfunction and damage. The mitochondria have been highlighted as both originators and targets of toxic products of lipid peroxidation in heart, especially under pathophysiological conditions [88]. Hence, it is surmised that the reactive products of lipid peroxidation can also cause the mitochondrial dysfunction and damage in the respiratory tract including lung thus contributing to the respiratory and lung diseases (Fig. 6.2). Linoleate hydroperoxide (LOOH) has been shown to inhibit the agonist-induced O_2^- formation and cause the mitochondrial membrane alterations in the rat alveolar macrophages [89]. Hence, it is possible that PUFA hydroperoxides such as the LOOH commonly formed in the alveolar macrophages by either enzymatic or nonenzymatic oxidation of linoleate of the cellular membranes may be involved in the macrophage-mediated lung disorders and diseases. Pulmonary surfactant has been shown to attenuate the LOOH-induced lipid peroxidation in the isolated rat lung mitochondria suggesting the role of lipoperoxidative damage of the lung

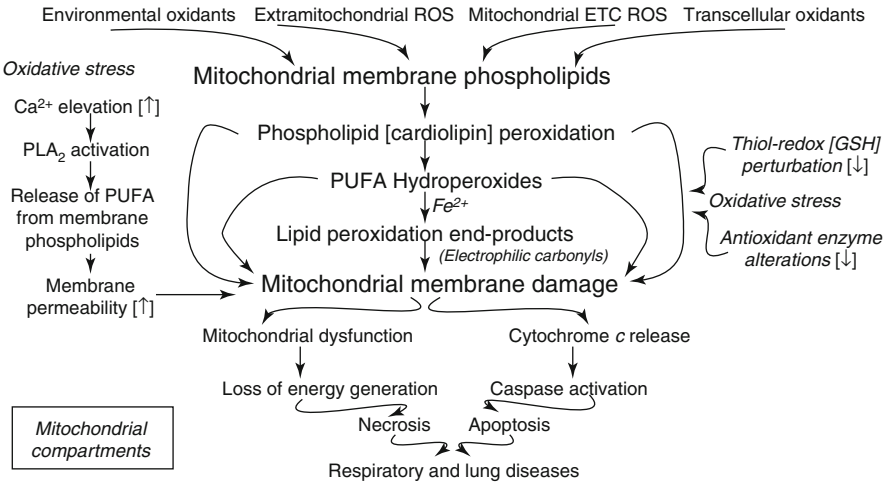

Fig. 6.2 Role of eicosanoids in causing mitochondrial damage and apoptosis in lung cells that are involved in respiratory and lung diseases

mitochondria and its protection by the surfactant proteins associated with acute respiratory distress syndrome (ARDS) [90]. The air pollutant and cigarette smoke constituent acrolein (a strong electrophilic and toxic α,β-unsaturated aldehyde) has also been shown to be formed from PUFA peroxidation in the cells that is involved in the pathogenesis of several diseases including the respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD), lung cancer, and cystic fibrosis [91–93]. In the human small airway epithelial cells (HSAEpCs), acrolein has been demonstrated to induce cytotoxicity and apoptosis through ROS generation, oxidative stress, apoptotic signaling pathway activation, and cytochrome *c* release from the mitochondria [93]. The acrolein-induced damage of HSAEpCs has been shown to be protected by inhibiting aldose reductase, an enzyme that is involved in glucose metabolism possibly through suppressing the reactivity of acrolein with GSH attenuating the formation of acrolein-GSH adducts. From this study it is clear that the PUFA lipid peroxidation product acrolein causes damage to the airway epithelial cell mitochondria leading to the apoptotic cell death. This is also supported by another study which has demonstrated that acrolein causes cytotoxicity and the mitochondria-mediated apoptosis in human lung cancer A549 cells [94].

The role of oxLDL has been recognized in the pathogenesis of atherosclerosis and associated cardiovascular diseases. However, oxLDL has also been identified as an inducer of the mitochondrial ROS generation in the vascular endothelial cells (ECs), and the underlying mechanism is yet to be established [95]. Thus, the metabolically formed and highly reactive peroxidized oxLDL is capable of inducing ROS generation in the mitochondria that has been shown to be associated with the vascular EC dysfunction, activation, and inflammation in conjunction with the pathogenesis of atherosclerosis. Although it is farfetched, it is probable that oxLDL could be involved in the pathogenesis of certain respiratory and lung diseases

through the generation of ROS in the mitochondria of lung vasculature which warrants further in depth studies.

Lipid oxygenases such as cyclooxygenases (COXs) and lipoxygenases (LOXs) are known to oxidize PUFAs, especially arachidonic acid released from the membrane PLs upon the action of phospholipase A_2 leading to the formation of bioactive eicosanoids including the prostaglandins (PGs), PUFA hydroperoxides (PUFA-OOH), and leukotrienes (LTs). These eicosanoids exert diverse physiological actions in the cells leading to both cell injury and cytoprotective responses. However, it is beginning to emerge that the eicosanoids also exhibit profound mitochondria-affecting actions in the lung. In the A549 lung cancer cell line, it has been shown that the PGs apparently induce apoptosis through the regulation of expression of the PGE_2 receptors (EP receptors) and $PGF_2\alpha$ receptors (FP receptors), especially the former being the only one observed in the mitochondria and both types of receptors being present in the plasma membrane [96]. This study demonstrates that the COX-generated PGs are involved in the lung cancer cell apoptosis through regulation of expression of the EP and FP receptors in the cell and mitochondrial membranes. COX-2, an isoform of the COX family of enzymes, is known to regulate the tumor growth, and therefore a study has been conducted to demonstrate the localization of COX-2 in several types of cancer cells including the lung cancer A549 cell line [97]. This study reveals that the presence and co-localization of COX-2 and heat-shock protein 60 (HSP60) in the mitochondria of A549 lung cancer cells. Furthermore, this study suggests that COX-2 may be involved in the cancer cell resistance to apoptotic death. The cyclopentenone class PGs (PGJ_2s), especially the 15-deoxy-$\Delta 12{,}14$-prostaglandin J2 (15d-PGJ_2), originating from dehydration of the COX-generated PGD_2, are highly bioactive and mediate important cellular functions including progression of cell cycle, expression of HSP, cell growth and differentiation, anti-inflammatory response, cytoskeletal alterations, changes in redox status, protein synthesis, and apoptosis [98]. More importantly, the elevated levels of 15d-PGJ2 formed from the COX-catalyzed arachidonic acid metabolism during several disease conditions including inflammation has been recognized [98]. The cyclopentenone PGJ_2s including 15d-PGJ_2 contain the α,β-unsaturated carbonyl group and are electrophilic in nature, and therefore they form covalent adducts (Michael addition) with nucleophiles (-SH group) including cysteine and GSH in a non-receptor mechanism leading to the regulation of redox-dependent cellular signal transduction cascades [98]. In the human non-small cell lung carcinoma cells (A549 cells), 15d-PGJ_2 has been observed to induce apoptosis through the mitochondrial cytochrome *c* release [99]. Thus, this study reveals that the cyclopentenone PG induces apoptotic cell death in the human lung A549 cancer cells through activation of the mitochondrial pathway of apoptosis.

The LOX-generated eicosanoids have been shown to be bioactive and elevated during the inflammatory respiratory diseases such as asthma [100]. In the human lung A549 cells, interleukin-4 (IL-4) has been shown to cause the upregulation of 15-LOX that catalyzes the formation of 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE), which induces apoptosis in the cells upon binding to the peroxisome proliferator-activated receptor γ (PPARγ). This study also reveals the involvement

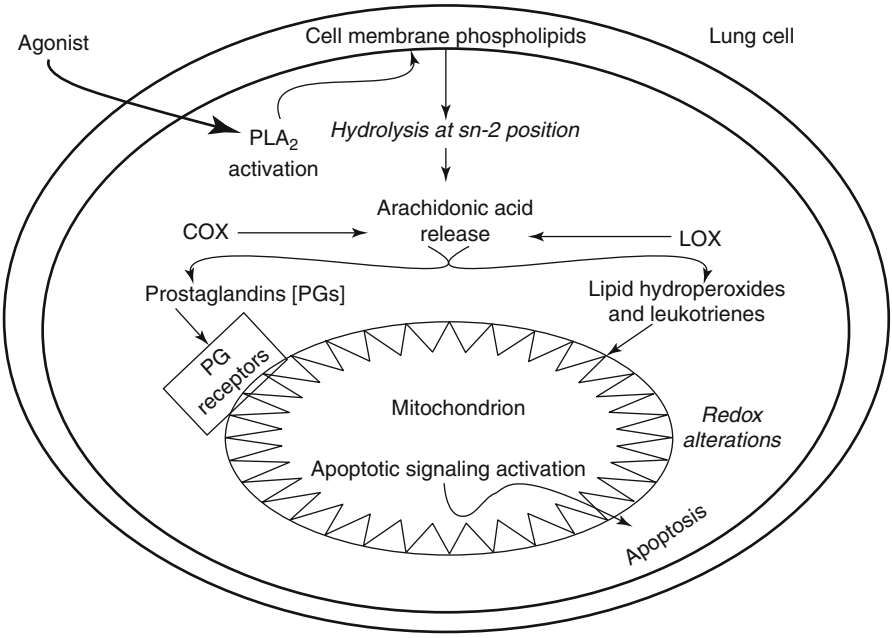

Fig. 6.3 Mechanism of ROS-induced membrane lipid peroxidation leading to mitochondrial dysfunction, necrosis, and apoptosis in lung cells as underlying mechanism(s) of respiratory and lung diseases

of mitochondria (cytochrome *c* release, translocation of the cytoplasmic Bax protein to mitochondria) in the IL-4-induced apoptotic death of the A549 cells through the actions of 15(S)-HETE which may have implications in the pathogenesis of chronic asthma [100]. Exposure of mice and cultured alveolar macrophages to inhalable carbonaceous airborne particles with high electron spin density (free radical characteristics) have shown inflammation, oxidative stress, and mitochondrial abnormalities in the alveolar macrophages in vivo, which are associated with enhanced NO production in lavage fluid of the lung and elevated nitrotyrosine formation in the lung tissue [101]. More interestingly, this study reveals that the alveolar macrophages in culture upon exposure to the carbonaceous airborne particles with high electron spin density exhibit elevated levels of secreted LTB_4, an eicosanoid formed from the LOX-catalyzed metabolism of arachidonic acid, suggesting the association of airborne particle-induced mitochondrial damage and LTB_4 secretion in the alveolar macrophages. Also, the role of LOX-, COX-, and cytochrome P 450-generated eicosanoids in induction of the mitochondria-mediated apoptosis is emphasized [102]. Taken together, the above highlighted studies have clearly demonstrated that the PUFA peroxidation products generated by nonenzymatic and enzymatic catalysis are bioactive in causing the mitochondrial dysfunction that leads to the cellular and tissue damage of the lungs with implications in the pathogenesis of respiratory diseases (Figs. 6.2 and 6.3).

Mitochondria-Targeted Free Radical Scavengers and Antioxidants and Protection Against Lipid Peroxidation

The search for effective free radical scavengers and antioxidants to inhibit oxidative stress and lipid peroxidation is a continuous and evolving endeavor in the arena of oxidant biology [11]. The use of water-soluble compounds (e.g., vitamin C and thiol protectants such as NAC) and lipid soluble antioxidants (vitamin E and phytochemical polyphenols and natural products) has been in practice to intervene oxidative stress and protect against oxidant injury in biological systems including the oxidative lung damage [103–106]. However, targeting specific cellular sites such as mitochondria which are both sources and targets of ROS and oxidative stress with effective free radical scavengers and antioxidants is a challenging task. In this regard, the cell-permeable Szeto-Schiller (SS) peptides have emerged as the novel mitochondria-targeted peptide antioxidants which could offer promise in protecting against the oxidant-induced mitochondrial lipid peroxidation and damage [107–109]. In order to effectively target the specific mitochondrial membranes and compartments for effective antioxidant action and protection against the ROS production and oxidant-induced CL peroxidation in mitochondria and apoptosis, strategies such as designing and synthesizing nitroxide-natural product conjugates and nitroxide-gramicidin conjugates have been ingeniously proposed [110]. Especially, the delivery and targeting mitochondrial locations (compartments) precisely with antioxidants (small molecules and enzymes) with the use of efficient triphenylphosphonium and hemi-gramicidin S cargoes have been emphasized [111]. However, dual action compounds that are equally effective as antioxidants (scavenging free radicals and inhibiting lipid peroxidation) specifically in the mitochondria and as apoptosis inhibitors are an unmet need for the therapeutic intervention of oxidant lung damage and oxidative stress-mediated respiratory and lung diseases wherein the mitochondria are at the epicenter.

Conclusions

Currently, there is convincing evidence in favor of the role of lipid peroxidation in the lung mitochondria as an important factor in mediating the lung damage and diseases. One noteworthy feature of the lung mitochondrial lipid peroxidation is the oxidative deterioration of the unique and most crucial phospholipid of the inner mitochondrial membrane, CL, which leads to the apoptotic death of lung cells (endothelial and epithelial). Antioxidant drugs targeted to specific mitochondrial compartments with dual action of scavenging free radicals and inhibiting lipid peroxidation and blocking apoptosis should be considered for effective therapeutic intervention of oxidant-induced mitochondriopathy in the pathogenesis of respiratory and lung diseases.

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Chapter 7
The Impact of DNA Damage on Epithelial Cell Maintenance of the Lung

Lidza Kalifa and Michael A. O'Reilly

Abstract The primary function of the lung is to facilitate the exchange of oxygen and carbon dioxide between air and blood and to exclude or defend against infectious agents and other airborne pollutants. As such, the respiratory epithelium is under constant attack by reactive oxygen species derived from metabolic respiration and the inflammatory response to pathogens in the airway. While reactive oxygen species can damage all macromolecules, oxidative damage to DNA is of great importance because it can affect how cells and hence organs function. DNA lesions activate a family of phosphatidylinositol-3 kinase-related kinases (PIKKs) that phosphorylate numerous substrates, including the tumor suppressor p53, which is critically important for maintaining genome integrity. While oxidized DNA is historically thought to be detrimental to cell function, emerging evidence suggest that it may also be an important post-replication modification that controls gene expression. Understanding how oxidation of nuclear and mitochondrial DNA affects cell function could provide new opportunities for treating lung diseases attributed to oxidant injury to the respiratory epithelium.

Keywords Reactive oxygen species • DNA damage • Mitochondria • Nucleus • Cell signaling

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Introduction

The primary function of the lung is to exchange oxygen in the air with carbon dioxide produced during metabolic processes. It develops as a dichotomously branching organ that in humans terminates in 300 million alveoli, which provide the extremely large surface area needed to efficiently exchange large amounts of gas. The mammalian lung is composed of over 40 different cell types, including several specific types of epithelial, vascular, lymphatic endothelial, and immune cells, which are regionally localized throughout the organ [1–6]. These cells collectively give the lung its characteristic branched structure lined by an 80-m^2 sheet of epithelial cells. This epithelial lining provides the first line of defense against inhaled substances including respiratory pathogens (bacteria, virus), allergens (mold, animal dander), and environmental air pollutants (cigarette smoke, ozone, particulate matter). Leukocytes recruited to the site of epithelial injury can cause additional damage as they attack foreign substances. Efficient identification and removal of foreign substances from the lung is therefore crucial for maintaining pulmonary function. Equally important is the ability to remove damaged cells and replace them from progenitor pools. Failure to execute these processes properly has been associated with a variety of pulmonary diseases, including chronic obstructive pulmonary diseases (COPDs), idiopathic interstitial pneumonia, autoimmune-related fibrosis, and asthma [6].

Because the respiratory epithelium undergoes aerobic respiration, exchanges oxidant gases, and provides a barrier against inhaled pathogens or pollutants, it is exposed to higher levels of reactive oxygen species (ROS) than most other cells or organs [7]. ROS are produced intrinsically as cellular respiration generates ATP. However, electrons shuttling down the electrochemical gradient frequently escape the electron transport chain at complexes I, II, and III and reduce oxygen to a superoxide anion ($O_2^{\cdot -}$) [8–16]. Superoxide anions can subsequently be converted to hydrogen peroxide (H_2O_2) and a highly reactive hydroxyl radical ($OH\cdot$) [8, 9]. Intrinsically produced ROS play an important role in regulating cellular functions, including proliferation, apoptosis, transcription, and defense against pathogens. ROS may also be produced when cells are damaged or by inflammatory cells recruited to sites of damage. For example, vast amounts of superoxide anion are produced when neutrophils become activated in the lung [17]. ROS are also produced by environmental and life-style choices. Tobacco smoke, viral infections, diesel exhaust, ozone, biocides and pesticides are examples of extrinsic environmental stimuli contributing to ROS production [18]. Hence, the respiratory epithelium is under constant attack by intrinsic as well as extrinsic forms of ROS.

In order to protect against ROS-induced damage, cellular defense mechanisms exist to detoxify ROS and remove damaged cells. Nonenzymatic antioxidants such as vitamin E, vitamin C, and glutathione protect by trapping or reducing ROS. Superoxide dismutases (SODs), catalase, thioredoxin, and glutathione peroxidase are some of the enzymes which function to significantly reduce the concentration of ROS in the cell by enzymatically converting ROS to less toxic molecules.

Furthermore, some of these enzymatic antioxidants have been shown to increase perinatally to prepare for the shift from low ambient oxygen levels in utero to atmospheric oxygen at birth [19–22]. Antiapoptotic pathways may provide another line of defense by allowing more time for oxidative damage to be repaired. Bcl-XL may be one example of such a pathway because conditional loss in the respiratory epithelium results in perinatal lethality in some mice [23].

Oxidative damage to macromolecules, including lipids, carbohydrates, proteins, and nucleic acids, occurs when ROS exceeds the potential of detoxifying agents [24]. While oxidative damage of any molecule can be injurious, oxidative damage to DNA is arguably of great importance because maintaining DNA fidelity is essential for ensuring cell survival and function. Indeed, DNA lesions that are not effectively repaired may result in mutations which are a hallmark of cancer [25]. ROS contributes to oxidation, nitration, depurination, methylation, and deamination of DNA bases [26]. Oxidized base adducts can form either directly in DNA or in the free deoxynucleotide pool and subsequently incorporated during DNA replication. Apurinic/apyrimidinic (AP) sites are also generated by endogenous oxidative stress at a rate estimated at greater than 10,000 bases/d in mammalian cells [27–29]. In fact, in vitro studies using A549 adenocarcinoma human alveolar epithelial cells illustrate a time-dependent increase in 8-oxo-2′deoxyguanosine (8oxoG) as well as single- and double-strand DNA breaks following exposure to elevated oxygen (hyperoxia) demonstrating the correlation between oxidative stress and DNA lesions [30, 31]. Furthermore, lung tissue has a marked increase in 8oxoG and DNA strand breaks after exposure to hyperoxia [32, 33]. Oxidized lipids can also damage DNA [34]. The indiscriminate production of ROS by inflammatory cells such as polymorphonuclear (PMN) cells has been shown to increase 8oxodG in respiratory epithelial cells [35]. The antineoplastic drug bleomycin causes DNA double-strand breaks in alveolar epithelial type I cell and microvascular endothelial cells, resulting in death of these cell types [36, 37]. Oxidized nucleotides have been detected in fibroblasts exposed to cigarette smoke [38, 39]. Regardless of the source, if not dealt with, damaged DNA may block replication and the transcriptional machinery leading to numerous cellular consequences, including cell death or cancer. In the lung, severe and repetitive epithelial injury has been linked to pulmonary fibrosis and emphysema [40]. In fact, oxidized DNA has been found in pulmonary epithelium of patients with severe emphysema [41]. Thus, understanding how pulmonary epithelial cells respond to oxidative DNA damage may provide new opportunities for treating lung disease.

Mitochondrial DNA Is Different than Nuclear DNA

The mammalian nuclear genome consists of 24 linear chromosomes approximately 26,000 genes. Nuclear DNA is wrapped around a core of histone proteins forming a nucleosome and further compacted by inter-nucleosome interactions resulting in an

organized, dynamic chromatin structure [42]. Eukaryotic cells also contain DNA in the mitochondrial compartment. Mammalian mitochondrial DNA (mtDNA) is a double-stranded circular molecule of approximately 16.6 kb in size and is present in 10^3–10^4 copies per cell [43]. The majority of proteins that function in mitochondria are encoded in the nuclear genome. However, mtDNA encodes a subset of components required for oxidative phosphorylation and ATP synthesis, as well as the tRNAs and rRNAs essential for expression of these genes. Although previously thought to be "naked", mtDNA is also packaged into higher-order nucleoid structures by "histone-like" proteins. It is estimated that each nucleoid contains 6–10 mitochondrial genomes [44]. While packaging mechanisms differ between nuclear and mitochondrial DNA, they both serve to compact and coordinate DNA metabolic processes, such as replication and transcription. Additionally, chromatin and nucleoid structures are thought to provide some protection from genotoxic stress by concealing the DNA from the environment.

Due to the environment and more loosely organized structure, it has been suggested that mtDNA is 3–10 times more susceptible to oxidative DNA damage than nuclear DNA [45–47]. In support, 8oxoG staining has been co-localized with mitochondrial structures indicating the majority of oxidized guanines are in mtDNA [32, 48, 49]. Furthermore, several lines of evidence support the idea that chronic oxidative damage to mtDNA may result in degradation causing a decrease in mtDNA copy number [49–51]. Consistent with this theory, ROS may be a key modulator of mtDNA copy number control [52–54]. Persistent mtDNA lesions and a decrease in mtDNA copy number result in drastic decreases in oxidative phosphorylation and reduced mitochondrial reserve capacity [51]. While mtDNA may be more prone to DNA damage, cells contain thousands of heteroplasmic copies of the mitochondrial genome and a mutational threshold must be exceeded for a phenotypic outcome. In fact, it has been estimated that greater than 50 % of mitochondrial genomes must be mutated in order to measure a decrease in mitochondrial function [55, 56]. Due to the differences in size, copy number, organization, and sequence context, it remains to be determined whether cells respond the same or differently when the mitochondrial versus nuclear genomes are damaged.

The Cellular Response to DNA Damage

The cellular response to nuclear DNA damage is a complex series of signal transduction pathways consisting of sensors that recognize DNA lesions, transducers that signal DNA is damaged, and effectors that control how the cell responds to the damage. This DNA damage response (DDR) initiates a cascade of events that function to recognize DNA aberrations and induce cellular growth arrest, DNA repair, or, in the case of irreparable damage, cell death. One can envision how this signaling cascade evolved to ensure that the two copies of each nuclear-encoded gene were faithfully maintained. But, it is less clear whether such a complex network is necessary

to ensure that the multi-copy mitochondrial genome is also maintained. Emerging evidence suggests the existence of a mitochondrial retrograde signaling system in which signals emanating from mitochondria traffic to the nucleus where they affect transcription of nuclear-encoded genes regulating mitochondrial function.

DNA Damage Response: Sensors

DNA damage sensors recognize aberrant DNA structures and initiate a signaling cascade. While this remains an open area of study, poly (ADP-ribose) polymerase (PARP) and DNA-dependent protein kinase (DNA-PK) have long been proposed as DNA damage sensors due to their ability to bind DNA strand breaks. However, evidence suggests that these proteins do not recognize other DNA structural abnormalities. In the recent years, genetic and biochemical studies in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* have suggested that DNA damage sensors include a heterotrimeric clamp-like complex of Rad1, Rad9, and Hus1 (911), as well as Rad17-replication factor C 2–5 (RFC) clamp loader complex [57, 58]. These proteins are highly conserved structurally and functionally from yeast to humans [59]. Furthermore, ablation of Hus1 in mice results in embryonic lethality and impaired DNA damage signaling in response to genotoxic stress [60–62]. Other studies have suggested that there are several proteins that may act as sensors due to the wide range of DNA structural modifications resulting from different DNA damaging agents. Some additional candidate sensors include BRCA1, MRE11-RAD50-NBS1 complex (MRN), and MSH2/6 and MLH2 due to their abilities to bind and recognize DNA lesions and activate the appropriate signaling cascade [63–68].

The DDR sensors described above have been identified due to roles in the nuclear DDR or nuclear genomic instability. Evidence of mtDNA repair suggests that lesions in mtDNA are recognized and repaired. However, whether a DDR pathway exists in mitochondria remains unclear. Current studies propose co-localization of nuclear DDR sensors in the mitochondrial compartment. It has been reported that a fraction of PARP1 co-localizes to mitochondria and is vital for mtDNA maintenance [69]. Likewise, the MRN homologs in *Saccharomyces cerevisiae* (Mre11-Rad50-Xrs2) have been implicated in maintaining mtDNA [70]. Whether these proteins or others initiate a canonical DDR signaling cascade as for nuclear DNA damage remain to be determined.

Despite the importance of sensors to detect DNA damage and the knowledge that the respiratory epithelium is under constant oxidative attack, very little is known about how sensors function in lung disease. Increased expression of Rad9 has been detected in type II epithelial cells of mice or A549 cells treated with bleomycin or lipopolysaccharide [71]. Since loss of Rad9 increased sensitivity of A549 cells to bleomycin, elevated levels of Rad9 is protective. Furthermore, siblings with ataxia-telangiectasia-like disorder (A-TLD) resulting from mutations in coding region of MRE11 developed lung adenocarcinomas [72]. People inflicted with Nijmegen

breakage syndrome (NBS) due to hypomorphic mutations in NBS1 have predisposition to respiratory tract infections frequently resulting in respiratory failure and death [73]. These limited examples support the idea that DDR sensors may be of vital importance for maintaining lung tissue homeostasis.

DNA Damage Response: Transducers

Although the proteins that sense DNA damage are not well understood, a lot more is known about the phosphatidylinositol-3 kinase-related kinases (PIKKs) that function to transduce the response to effector molecules controlling the cellular response to DNA damage. Humans have six PIKKs, of which four are involved in the DNA damage response (ATM, ATR, DNA-PKcs, Smg1), one is involved in nutrient sensing (mTOR), and one functions as a transcriptional co-regulator of gene expression (TTRAP) [39]. ATM (ataxia-telangiectasia mutated) and ATR (ataxia- and Rad3-related) proteins are the primary transducers of the response to DNA lesions and will be discussed in more detail. DNA-PKcs interacts with Ku proteins to promote nonhomologous end joining of DNA double-strand breaks. Smg1 (suppressor of morphogenesis in genitalia) regulates nonsense-mediated mRNA decay as well as the response to DNA damage. mTOR (mammalian target of rapamycin) controls protein synthesis in response to periods of cell starvation or stress. TRRAP (transformation/transcription domain-associated protein) is an adaptor protein thought to be involved in epigenetic modification of genes because it is found in multiprotein complexes containing histone acetyltransferase. ATM, ATR, DNA-PKcs, and Smg-1 are all activated by DNA damage and activate downstream signaling events by phosphorylating proteins on serine/threonine residues [74].

Among these members, ATM represents the primary responder to DNA damage induced by ionizing radiation (IR) and radiomimetics, or agents resulting in double-strand breaks (DSB) [75]. ATR is activated by a broader range of genotoxic stressors, particularly those that cause replication fork stalling [76]. This may explain why deletion of ATR, but not ATM, results in embryonic lethality [77]. While a connection between ATR and mitochondria has yet to be identified, links between mitochondrial function and ATM have suggested that ATM may serve as a mitochondrial transducer. Recent localization of ATM to the mitochondrial compartment in normal human fibroblasts further supports this theory [78]. Investigation of ATM-deficient cell lines showed reduced mitochondrial respiratory capacity and diminished cytochrome c oxidase activity [79, 80]. In mouse thymocytes, loss of ATM altered mitochondrial morphology resulting in swollen, poorly organized cristae as well as increases in mitochondrial mass and mitochondrially produced ROS [78]. Whether these outcomes reflect a failure to transduce mtDNA damage or non-DNA damage property of ATM remains to be determined.

Deficiency or mutation of ATM kinase has been linked to the rare autosomal recessive disorder ataxia-telangiectasia (A-T), which results in progressive neurological degeneration, predisposition to cancer, acute sensitivity to ionizing radiation,

and a compromised immune response [81–83]. Interestingly, chronic lung disease develops in a significant proportion of people with A-T deficiency suggesting that ATM may be crucial in cellular lung repair or regeneration [84]. Moreover, morbidity and mortality of A-T patients is commonly associated with respiratory diseases, particularly after a viral infection [85–87]. How loss of ATM leads to lung disease is not known. Respiratory viruses cause DNA double-strand breaks as they integrate into the nuclear genome. Additional damage is likely to occur when neutrophils and $CD8^+$ T cells attack virally infected cells. Unlike ATM, deficiencies in ATR have not yet been identified in lung disease. However, mutations in ATR have been implicated in the rare autosomal recessive disease Seckel syndrome and cutaneous telangiectasia and cancer syndrome (FCTCS). Despite the lack of lung disease in ATR deficiency, in vitro studies using A549 cells suggest that both ATM and ATR are activated in response to hyperoxia, a toxin that damages DNA [88, 89]. Given their essential role in transducing the DDR, these PIKKs are likely to play an important role in how the respiratory epithelium responds to various forms of genotoxic stress.

DNA Damage Response: Effectors of the Cellular Response

The function of transducers is to activate effectors, which can induce numerous cellular responses including cell cycle arrest, DNA repair, cell death, and, in general, maintenance of cellular health. Although there are a plethora of proteins that can be modified via ATM and ATR, arguably, one of the most vital effectors is the tumor suppressor protein p53. p53 is a sequence-specific transcription factor that is frequently mutated in cancer and is best known for its ability to inhibit cell growth and stimulate apoptosis [90]. It contains an amino-terminal transactivation domain, a central DNA binding domain, and a carboxy-terminal tetramerization domain. p53 normally exists in an inactive state and maintained at low levels by the ubiquitin-dependent ligases Mdm2, PirH2, COP-1, and CHIP that target p53 for degradation [91]. It is posttranslationally modified on more than 36 amino acids during normal homeostasis and under cellular stress. Posttranslational phosphorylation, acetylation, and ubiquitinylation of p53 are perhaps the most widely studied modifications; however, p53 has also been reported to undergo sumoylation, neddylation, methylation, and glycosylation [92]. Acetylation occurs at several sites and functions to stimulate chromatin remodeling. Phosphorylation occurs at dozens of sites, of which phosphorylations on Ser15 (Ser18 in mice), Thr18, and Ser20 (Ser23 in mice) are conserved between humans and urochordates, and Ser392 is conserved among vertebrates. DNA damage leads to ATM-, ATR-, and Smg1-dependent phosphorylation of p53 on Ser15. ATM and ATR also phosphorylate checkpoint kinase (Chk) 2 and 1 respectively, and these in turn phosphorylate p53-serine 20 [93–97]. Interestingly, knock-in mice containing serine to alanine substitutions at amino acids 20 or 23 display fairly normal p53 response to stress, whereas compound Ser18/Ser23 mutant mice show some loss of function effects [98]. The evolution of multiple sites of modification may allow for integration of diverse signals that lead to a common effector function.

Cell Cycle Arrest

One of the primary functions of the DDR pathway is to induce cell cycle arrest, which prevents damaged DNA from being replicated and fixed in the genome. The cell cycle is divided into two stages, interphase (consisting of G1, S, and G2) and M phase. During M or the mitotic phase, the replicated chromosomes are segregated into separate nuclei, and cytokinesis results in the formation of a daughter cell. Interphase consists of G1, a gap phase in which cells prepare for DNA replication; S or synthesis phase in which the DNA is replicated; and G2, a second gap phase in which the cell prepares for division. These processes are highly coordinated and monitored by checkpoints to ensure accuracy and completion of one phase prior to transitioning to the next. p53 promotes G1 and G2 arrest of damaged cells through multiple mechanisms. This includes transcriptional stimulation of the cell cycle inhibitor p21 controlling G1 exit, suppression of proliferating cell nuclear antigen required for S phase progression, and repression of cyclin B1 required for G2 phase exit [99–101].

DNA Repair

Cell cycle arrest may also allow more time to repair damaged DNA, which is clearly necessary to restore DNA integrity and normal cell function. There are numerous repair pathways responsible for repairing specific DNA lesions, including mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER), double-strand break repair (DSBR), and DNA damage tolerance involving translesion DNA polymerases. Since oxidative DNA damage can result in base damage, as well as DNA strand breaks, multiple repair pathways are required to repair the various lesions. In both the nuclear and mitochondrial compartments, BER is the predominant pathway to repair oxidative base lesions. This pathway involves recognition and excision of the damaged DNA base(s), insertion of nucleotides, and subsequent ligation. BER can repair one nucleotide as in short patch (SP) or several nucleotides as in long patch (LP) repair. Recognition and excision of damaged bases is performed by DNA glycosylases which hydrolyze the N-glycosidic bond between the base and the deoxyribose sugar generating an apurinic/apyrimidinic (AP) site. Cleavage of the AP site by an AP endonuclease generates a substrate that can be extended by a polymerase. Ligase catalyzes phosphodiester bond formation to join the DNA strand and completes both SP and LP-BER [102, 103]. Here again, p53 plays an important role in facilitating DNA repair by interacting with APE/Ref-1 and DNA polymerase beta [104, 105].

Due to the complexity of many DNA lesions, repair frequently requires the interplay of different repair pathways. Additionally, the temporal and spatial regulation of DNA repair enzymes is critical for proper cellular maintenance. In fact, a study of normal bronchial epithelial cells collected from lung cancer versus non-lung cancer subjects suggests that susceptibility to lung cancer may correlate with extreme

deviation from the median expression levels of several DNA repair genes [106]. A key example is that while no diseases have been directly linked to deficiencies in BER, reduced activity of the glycosylase OGG1 has been identified as a major risk factor in sporadic lung cancer [107]. Also, chronic, incremental increases in ROS result in higher levels of the mitochondrial AP endonuclease (APE) which lower levels of mtDNA damage and increased repair rates after further oxidative damage [108]. Moreover, lung epithelial expression of Polymerase Lambda (Pol λ), involved in BER and DSBR via nonhomologous end joining, correlated with habitual cigarette smoking suggesting that Pol λ is important for repairing DNA damage induced by tobacco smoke [109].

Cell Death

Prolonged and extensive DNA damage may lead cellular senescence or cell death. Apoptosis is the orchestration of cellular death which is vital for homeostasis and development of multicellular organisms, protection against irreversibly damaged cells, and in senescence and aging [9]. There are several factors, both intrinsic and extrinsic, that can activate pathways resulting in apoptosis. In all cases, apoptosis is a regulated pathway, which involves the activation of numerous cysteine aspartic acid-specific proteases termed caspases. Activation of the caspase cascade ultimately results in cellular fragmentation which can be degraded by phagocytes [110]. The decline of mitochondrial membrane potential, constitutive opening of mitochondrial pores, arrest of oxidative phosphorylation, interruption of mitochondrial protein import, and leakage of cytochrome c into the cytoplasm have all been associated with apoptosis [9]. While apoptosis is a control pathway resulting in cell death, frequently oxidative stress results in necrotic death and a subsequent inflammatory response [111].

DNA Damage as a Modulator of Cellular Differentiation

There is going appreciation that the cellular response to DNA damage can also regulate cell differentiation. For example, DNA DSBs are created and repaired in pre-B cells as the antigen receptor undergoes rearrangement or mature antibodies are created upon antigen stimulation. DSBs within the immunoglobulin locus are repaired by nonhomologous end joining and involve ATM-dependent and ATM-independent signaling [112]. Interestingly, exposure of pre-B cells or germinal center cells to genotoxic agents activates the same differentiation program regulated by rearrangement of immunoglobulin genes [112, 113]. On the other hand, DNA damage accumulates in hematopoietic stem cells with age and may contribute age-related loss of function [114]. Surprisingly, p53 itself has been shown to both promote and inhibit differentiation of embryonic stem cells. Early studies showed how p53 suppressed growth and enhanced differentiation of mouse embryonic stem cells in response to

DNA damage [115, 116]. In this capacity, p53 inhibits growth of the cells while also directly suppressing transcription of Nanog, a gene required for stem cell renewal [117]. Recently, p53 has been shown to extend the cell cycle in G1 thereby allowing more time for the enhanced expression of microRNAs miR-34a and miR-145 to repress genes promoting stem cell phenotype [118]. Such findings support conclusions of a genome-wide screening study supporting a model wherein the DNA damage dependent production of p53 controls ES cell phenotype through activation of differentiation-associated genes and suppression of ES cell-enriched genes [119]. While most studies support the idea that DNA damage activates p53 that stimulates differentiation, basal levels of p53 appear to play an important role in self-renewal under nonstressed or damaged conditions [120]. Regardless, the notion that the DDR controls pluripotency and self-renewal of ES cells emphasizes the need to know whether it also controls differentiation of multi- or bipotential cells too. By controlling cell proliferation and differentiation, the DDR could conceivably be responsible for ensuring proper repair of the injured lung. Failure to do so could lead to hyperplasia of the epithelium, such as seen in bronchopulmonary dysplasia, or overexpansion of fibroblasts such as seen in idiopathic pulmonary fibrosis. This remains to be investigated.

DNA Lesions as a Regulator of Gene Expression and Differentiation

Although DNA modifications are often thought to be detrimental to normal cellular function or survival, recent studies suggest they play an important role and perhaps normal role in regulating gene expression. Post-DNA replication methylation of cytosines (5mC) by DNA methyltransferases is perhaps the best example of how modified DNA regulates gene expression [121]. 5mC occurs in CpG dinucleotides that are often found in stretches of DNA that termed CpG islands. In the mammalian genome, CpG islands are frequently associated with sites of transcriptional initiation, promoter regions. Hypermethylation of DNA generally correlates with transcriptional silencing, while hypomethylation generally stimulates transcription. This post-replication modification of DNA, along with modification of histone tails and noncoding RNAs, acts to epigenetically regulate gene expression. Epigenetic mechanisms have been linked to differentiation of stem cells, X-chromosome inactivation, carcinogenesis, and cognition. Epigenetic mechanisms can play a significant role in how the environment affects both lung development and disease, particularly disease later in life [122]. For example, maternal folate intake during pregnancy has been linked to asthma in children [123, 124]. Likewise, a high-methyl diet can increase airway hyperresponsiveness and inflammation in mice [125]. Intrauterine growth restriction in rats induces sex-specific methylation of histones along the PPARγ gene, a member of the nuclear family of transcription factor [126, 127]. Since alveolar simplification is seen in mice when epithelial expression of PPARγ is ablated, epigenetic suppression of PPARγ may be responsible for how intrauterine

growth restriction alters postnatal lung growth and development [128]. Examples such as these emphasize the growing importance of how post-replication modification of DNA profoundly effects normal lung development and function.

Emerging evidence suggests that oxidized DNA bases also affect expression of some genes. Hypoxia stimulates expression of many genes, including vascular endothelial growth factor (VEGF), which stimulates angiogenesis in an attempt to restore oxygen delivery in hypoxic tissues. Although this is mediated by the activation of the transcription factor hypoxia-inducible factor (HIF)-1α, ROS produced during hypoxia also cause nucleotide-specific oxidative modifications in the hypoxia response region of the VEGF promoter that enhance the binding of HIF-1α [129]. Consistent with these lesions enhancing binding, compacting the promoter with the histone deacetylase inhibitor trichostatin A prevented hypoxia-induced oxidative modifications in the VEGF promoter. Interestingly, the production of ROS during hypoxia was associated with perinuclear clustering of mitochondria, implying mitochondrial production of ROS functions as an epigenetic modifier of gene expression [130]. Oxidized purines have also been detected in the hypoxia response elements of other genes, including hemoxygenase (HO)-1 and endothelin (ET)-1 genes [131]. Lung tissues from patients with COPD also contain abundant oxidized purines in the hypoxia response elements (HRE) of these genes [132]. Given that oxidized bases are present in the promoter regions of hypoxia-responsive genes, it may seem less surprising that these regions also contain DNA repair enzymes. Hypoxia stimulates recruitment of Ref-1/APE to the HRE where it enhances high affinity binding of HIF-1 to DNA [133]. Ref-1/APE is a bifunctional protein that serves as a rate-limiting enzyme during base excision repair of oxidized DNA and as a redox regulator [134]. While the mechanism by which Ref-1/APE regulates HIF-1α activity is not fully understood, the redox functions of Ref-1/APE have been shown to modulate the oxidation state of other transcription factors. In thyroid cells, Ref-1/APE regulates transcriptional activity of the homeodomain containing protein TTF-1/Nkx2.1 through a critical cysteine 87 in the amino-terminal transactivation domain [135]. TTF-1/Nkx2.1 transcription can also be stimulated when it binds poly (ADP-ribose) polymerase (PARP) and inhibited when it interacts with thymine-DNA glycosylase [136]. Hence, oxidized DNA bases within promoters of oxidant-sensitive genes may function to epigenetically recruit DNA repair enzymes that modulate the actions of transcription factors recruited to that site. Because the lung is constantly under oxidative stress from exposure to oxygen or the inflammatory response to inhaled pollutants, using DNA repair enzymes to facilitate transcriptional activity of genes like TTF-1 involved in lung development and repair is conceptually appealing.

Potential Impact of Mitochondrial Dysfunction in Lung Disease

Since ROS-induced mitochondrial damage is correlated with a decline in mitochondrial function, increasing ROS production during development may be even more detrimental. Premature infants often require oxygen supplementation therapy at

birth for survival; however, hyperoxic exposure inhibits lung alveolarization leading to bronchopulmonary dysplasia (BPD) [119]. Since hyperoxia results in increased mitochondrial ROS production, increasing ROS-induced damage and mitochondrial dysfunction maybe linked to BPD. For over a decade, it has been evident that mitochondrial function is required for normal growth of pulmonary alveolar A549 epithelial cells in culture [120]. After 4 days of culturing in hyperoxic conditions, A549 cells displayed mitochondrial dysfunction assessed by decreased ATP production, mitochondrial membrane potential, and respiratory rate. An in vivo study supports the hypothesis that BPD results from abnormal pulmonary development caused by mitochondrial dysfunction [121]. High levels of oxygen in newborn mice inhibited cellular respiration via disruption of the electron transport chain and impaired oxidative phosphorylation. Mitochondrial fractions obtained from lungs of hyperoxia-exposed mice displayed a decrease in complex I activity suggesting the arrest in alveolar development correlated with decreased respiratory capacity. This was further supported by mice treated with the complex I inhibitor, pyridaben, which led to a delay in alveolar development similar to that of hyperoxia. These studies signify the importance of mitochondrial function during lung development.

p53 and Mitochondria

The role of p53 in regulating nuclear gene expression to induced mitochondrial-dependent apoptosis has been well studied [112–114]. However, recent studies have identified a novel role for p53 in maintaining mitochondrial genome integrity suggesting that p53 may also function as a mitochondrial effector [115–118]. Mitochondrial localization of p53 has been identified in various cell types without exogenous genotoxic stress and has been suggested to function along with the mitochondrial polymerase (Pol γ) to stimulate mtDNA replication [115, 118]. Under increased ROS-induced stress, the p53 accumulates in the mitochondrial compartment suggesting that ROS and possibly a mitochondrial DDR may signal to recruit p53 and protect mtDNA from induced mutagenesis [118]. Modulation of p53 in the mitochondria is of importance since overexpression of p53 results in decreased mitochondrial function and mtDNA copy number [117].

New Opportunities for Understanding and Treating Lung Disease

The response to DNA damage is very complex and yet clearly essential for maintaining the health of a cell and hence function of the organ. While all cells of the lung must deal with ROS-mediated DNA damage, individual cells are more or less likely to be damaged by inhaled pollutants or inflammatory cells because of their location within the lung and cell-restricted pattern of gene expression. For simplicity, this chapter was written with the assumption that all epithelial cells respond to

DNA damage in the same manner. But, that clearly is too simple. Moreover, it is not possible to discuss every possible way that epithelial cells become injured or how they might undergo repair. We apologize to those investigators whose research was not discussed in this review. By providing a broader view of how cells respond to nuclear and mitochondrial DNA damage, we hoped to stimulate discussion and interest in research aimed at investigating the role of DNA damage in the maintenance of the respiratory epithelium. We propose that the following areas provide new opportunities for understanding the cellular response to DNA damage and hence may offer new ways to treat lung disease.

1. Are individual types of respiratory epithelial cells more or less resistant to genotoxic stress? In other words, are progenitor cells more or less resistant to agents that damage DNA than terminally mitotic cells?
2. Do inhaled toxins damage nuclear and/or mitochondrial DNA?
3. Do epithelial cells that function as progenitor cells respond differently to DNA damage than cells thought to be more terminally mitotic? Does DNA damage to progenitor cells affect their ability to differentiate and repair the injured lung?
4. If antioxidant enzymes and antiapoptotic proteins function to protect the lung as it transitions to air at birth, could DNA repair enzymes or the DDR also mature perinatally?
5. If stem cells transfer mitochondria to injured epithelial cells, could they also be transferring damaged mitochondria, and if so, what are the consequences?

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Chapter 8
Mitochondrial Function in Lung Health and Disease

Luis Puente-Maestu and Jorge Chancafe-Morgan

Abstract The mitochondrion is a membrane-enclosed organelle found in most eukaryotic cells. In addition to supplying cellular energy, mitochondria are involved in a range of other processes, such as signaling and cell death. The mitochondrion is able to sense PO_2 throughout the progressive reduction of cytochrome *c* with increasing hypoxia. This information is converted into an increase in reactive oxygen species production at complex III that acts as cell signal. Such signal is believed to be responsible for phenomena such as hypoxic vasoconstriction, activation of HIF, and internalization of the sodium-potassium pump of the alveolar epithelium. Mitochondrial DNA mutations are frequent in cancer cells. These mutations lead to an impaired oxidative metabolism and to decreased susceptibility to apoptosis. These two features are needed for survival of the cancer cell. There are two major signaling pathways of apoptosis and one of them involves the mitochondria. Apoptosis plays an important role in most lung diseases in two different ways. First, failure to clear unwanted cells by apoptosis will prolong the inflammation; second, excessive apoptosis may cause diseases. Finally the skeletal muscle dysfunction associated with COPD involves the loss of oxidative capacity, mainly due to the loss of mitochondrial density.

Keywords Apoptosis • Mitochondria • Permeability transition pore • Electron transport chain • Reactive oxygen species • Hypoxic vasoconstriction • Cell signaling • Skeletal muscle dysfunction • Remodeling • HIF • Mitochondrial DNA mutations

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Abbreviations

AMPK	AMP-activated protein kinase
Apaf1	Apoptotic protease activating factor 1
ATP	Adenosine triphosphate
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
CD95	Cluster of differentiation 95
Cl	Chlorine
CoA	Coenzyme A
COPD	Chronic obstructive pulmonary disease
COX	Cytochrome c oxidase
CS	Citrate synthase
DNA	Deoxyribonucleic acid
ENa	Amiloride-sensitive sodium channels
FADD	Fas-associated death domain protein
Fas	FAS receptor (FasR) also known as apoptosis antigen 1
FasL	Fas ligand
H_2O_2	Hydrogen peroxide
HIF	Hypoxia-inducible factor
HO-1	Heme oxygenase 1
IMS	Intermembrane space
IPF	Idiopathic pulmonary fibrosis
mDNA	Mitochondrial DNA
MPT	Mitochondrial permeability transition
Na	Sodium
NRS	Nitrosative reactive species
O_2	Molecular oxygen
p53	Tumor protein 53
PHD2	Prolyl hydroxylase domain-containing protein 2
PO_2	Oxygen pressure
PTP	Permeability transition pore
roGFP	Mutated variant of green fluorescent protein
ROS	Reactive oxygen species
SDH	Succinate dehydrogenase
SMD	Skeletal muscle dysfunction
SOD	Superoxide dismutase
TNF	Tumor necrosis factor
TRADD	TNF receptor-associated death domain

Introduction

The mitochondrion is a membrane-enclosed organelle found in most eukaryotic cells [1]. These organelles range from 0.5 to 1.0 micrometer (μm) in diameter. Mitochondria generate most of the cell's supply of adenosine triphosphate (ATP), the source of chemical energy [2]. In addition to supplying cellular energy, mitochondria are involved in a range of other processes, such as signaling, cellular differentiation, cell death, as well as the control of the cell cycle and cell growth [2]. While several mitochondrial functions can be studied separately, one has to keep in mind that these organelles are indispensable parts of the cell and the observed mitochondrial functions or dysfunctions cannot be separated, and many times are determined, from the physiology or pathophysiology of cells they belong. In this chapter we will focus on those aspects of mitochondrial function that are related to lung health and disease.

Response to Hypoxia

Alveolar Epithelial Responses to Hypoxia

Alveolar epithelial cells contribute to the clearance of edema fluid from the alveolar space by actively transporting sodium away from alveolar fluid into the interstitium using amiloride-sensitive sodium channels (ENa) and an ATP-dependent sodium-potassium pump in the basolateral membrane [3]. The movement of Na (and also Cl by the electrostatic gradient generated) across the epithelium draws water osmotically from the alveolus [3]. Hypoxia triggers the internalization of the sodium-potassium pump, possibly as a protective mechanism to limit ATP utilization. While this might confer protection to the epithelial cell itself, it lessens the ability to clear alveolar edema and may have even fatal consequences for the whole organism. Hypoxia-induced reactive oxygen species (ROS) production [4, 5] leads to the phosphorylation and activation of the AMP-activated protein kinase (AMPK) [4, 6].

The alveolar epithelium, as many other cells in the lung, initiates transcriptional responses to hypoxia by activating hypoxia-inducible factor (HIF) a proliferator factor responsible for activating a wide range of genes [7]. The activated HIF is a heterodimer composed of an oxygen-regulated subunit (HIF-1α) and a constitutively expressed, oxygen-independent component (HIF-1β). A related transcription factor, HIF-2, is regulated in a similar manner [8]. The α-subunit is continuously expressed, but under normoxic conditions it is degraded by the ubiquitin-proteasome system within minutes [9]. In hypoxia the prolyl hydroxylase domain-containing protein 2 (PHD2) stabilizes HIF-1α [10]. A growing body of evidence indicates that mitochondria-derived ROS has a major role in controlling the activity of PHD2. Cells that lack functional electron transport chain are unable to stabilize HIF-1α in hypoxia,

indicating that the oxygen-sensing response requires functional mitochondria [11]. The functionality of complex III [12, 13] and cytochrome c [14] is required for the hypoxic stabilization of HIF-1α and HIF-2.

Hypoxic Pulmonary Vasoconstriction

Smooth muscle cells from pulmonary arteries constrict during acute hypoxia [15–17]. While several oxygen sensors have been proposed and maybe more than one mechanism exist [16], one likely model of oxygen sensing implicates a role for the mitochondria [15]. Using a technique that measures low oxygen tensions rapidly and with precision, it has been found that as the extracellular oxygen tension decreases from approximately 60 mmHg (8 kPa) to approximately 5 mmHg (0.7 kPa), the cellular oxygen consumption rate remains unchanged, yet cytochrome *c* becomes progressively more reduced [18–20]. Such compensation is not possible below oxygen pressure (PO_2) lower than 1 %, since at that point all the cytochrome *c* is completely reduced by the electrons coming downward from the respiratory chain that cannot be transferred to cytochrome *c* oxidase (COX) since COX itself does not have oxygen available at the needed rate. This observation means that COX is able to maintain a normal O_2 consumption rate independent of the cell PO_2 not because of a very high affinity, but because cytochrome *c* becomes increasingly reduced [18–20]. Thus, while the electron flux (and thus mitochondrial respiration) does not become limited over this range, the decrease in the cell PO_2 causes a progressive redox change in the electron transport chain, which provides a readout of the PO_2.

The mechanism by which this information is converted into a signal is apparently related to ROS (Fig. 8.1). The mitochondrial production of ROS seems to be paradoxically increased in hypoxia [21–25]. Hypoxic release of ROS is inhibited by substances that block the electron flux into complex III [11, 26]. By means of roGFP, a mutated variant of green fluorescent protein that can be trafficked to specific cellular compartments [27, 28], it has been shown in cultured pulmonary artery smooth muscle cells that in progressively hypoxic conditions the oxidation of the intermembrane space and cytosol gradually augmented; however, the opposite occurred inside the mitochondrial matrix [29]. This finding can only be explained if the ROS production of complex III (which site of ROS production points toward the intermembrane space and has a relative readily access to the cytoplasm [30]) is dependent of hypoxia, while the ROS production of complex I is independent of hypoxia [14, 21, 31]. The mechanisms by which ROS trigger hypoxic pulmonary vasoconstriction are not fully defined [32]. Low concentrations of hydrogen peroxide (H_2O_2) are enough to induce vasoconstriction in the intact lung, while the hypoxic contractile response is abolished by antioxidants and inhibitors of complex III [33–35].

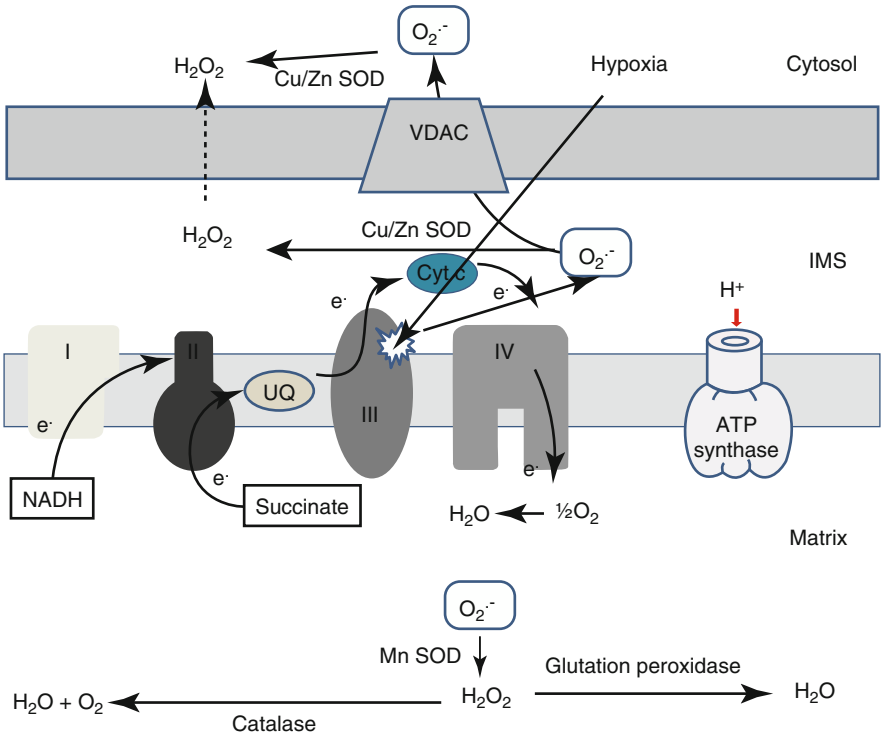

Fig. 8.1 Mitochondria as oxygen sensor. Mitochondrial reactive oxygen species generation at complex III of the respiratory chain. *IMS* intermembrane space, *SOD* superoxide dismutase

Mitochondria in Cancer

One constitutive metabolic characteristic of cancer cells is the upregulation of glucose metabolism and the dependence on anaerobic glycolysis for the synthesis of ATP, even in the presence of abundant oxygen. This phenomenon was first described by Warburg in 1930 [36], and it is accompanied by a durable shutdown of mitochondrial respiration that allows cancer cells to thrive in the hypoxic environments characteristic of solid tumors as the lesion grows progressively further from the blood supply [37, 38]. Moreover, this switch to aerobic glycolysis in cancer, the so-called Warburg effect, is the basis of positron emission tomography in which a glucose analog tracer (2-18fluoro-2-deoxy-D-glucose) differentiates between normal and tumor tissue by their avidity for glucose [36] (Fig. 8.2).

The mechanisms for the abnormal regulation of energy metabolism in cancer cells seem to be mediated by mitochondrial DNA mutations causing the impairment of the respiratory chain functioning [39, 40]. Some of these mutations inhibit

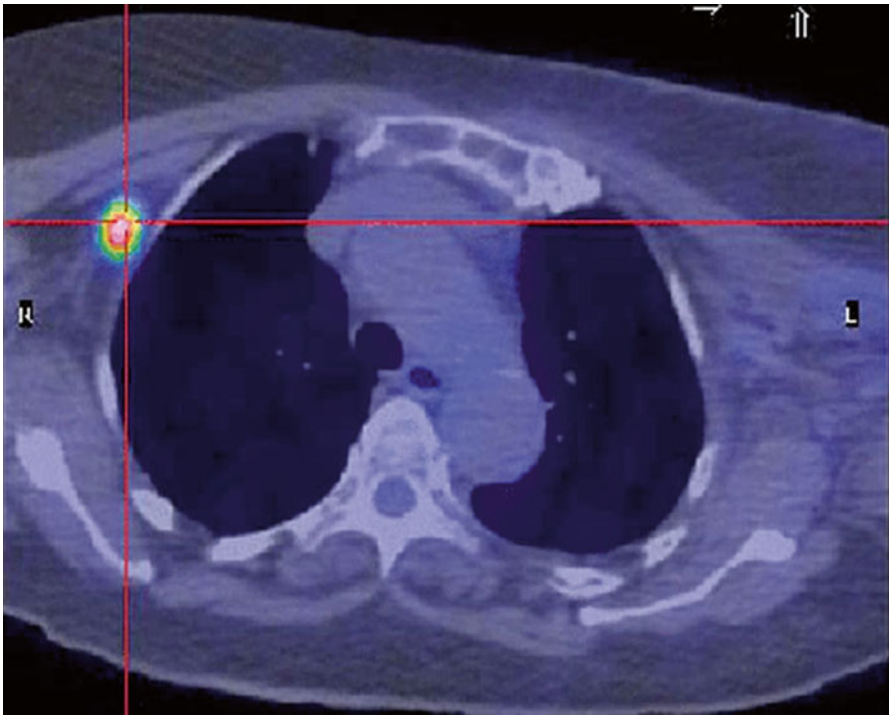

Fig. 8.2 Positron emission tomography. The switch to aerobic glycolysis in cancer, the so-called Warburg effect, is the basis of positron emission tomography in which a glucose analog tracer (2-18fluoro-2-deoxy-D-glucose) differentiates between normal and tumor tissue by their avidity for glucose

oxidative phosphorylation and enhance the production of ROS and thus promote tumor cell proliferation [39]. Another category of milder mutations could permit tumors to adapt to new microenvironments, especially when tumors progress and metastasize [39]. In most cases such mutations are acquired during or after oncogenesis; however, certain mitochondrial DNA mutations have been shown to provide a genetic predisposition to cancer development [39]. The known mechanisms by which these mutations lead to oncogenicity are dual [36]. In the first place the inactivation of the mitochondrial metabolic pathways endangers anaerobic glycolysis, but also certain mutations lead to the stabilization of HIF-1α and generate a pseudohypoxic state accompanied by HIF-dependent reprogramming of the metabolism toward aerobic glycolysis providing a gain of function in terms of the activation of hypoxia responses [37, 41]. The paradigm is in the case of some mutations affecting subunit B, C, or D of succinate dehydrogenase (SDH) causing pheochromocytoma and in the case of fumarate dehydrogenase leading to leiomyoma, leiomyosarcoma, or renal carcinoma [36]. The loss of activity of these enzymes results in the accumulation of succinate or fumarate in the cytosol, respectively. This, in

turn, favors the activation of HIF [37]. An alternative mechanism has been proposed for the mutations of SHD subunits. It suggests that when any other subunits of SDH are inactivated, the A subunit SDH causes an increase in ROS signaling, which stabilizes HIF-1α [41] by the mechanisms described above (see hypoxic pulmonary vasoconstriction). Some of these mutations of mitochondrial DNA might be useful tumor markers, with the advantage that are easier to determine since in the cell there are higher number of copies of the mitochondrial genes [42].

Another feature of mitochondria from cancer cells is their resistance against the induction of mitochondrial permeability transition (MPT), a process that mediates the intrinsic pathway of apoptosis [43–45]. In cancer cells MPT is regulated by proteins from the Bcl-2 family [46], the proteins forming the permeability transition pore (PTP) complex [47], proteins that affect mitochondrial dynamics (fusion and fission) [48, 49], and transcription factors (such as the tumor suppressor protein p53) [50]. MPT inhibition resulting in disabled apoptosis is important for the development of tumors [51]. Interestingly progression and resistance to therapy due to continued smoking may be due to the effects of nicotine on its ability to inhibit chemotherapy-induced apoptosis in lung cancer cells [45]. Thus, the mitochondrial alterations have clinical interest and are being the target of novel anticancer therapies [9, 36, 52] illustrating how the slow accumulation of fundamental knowledge eventually generates medically exploitable information.

Epithelial Cell Apoptosis and Lung Remodeling

Apoptosis may play an important role in lung diseases in two different ways. First, failure to clear unwanted cells by apoptosis will prolong the inflammation because of the release of their toxic contents and also delay repair processes [53]. Apoptotic cells should be quickly recognized and ingested by phagocytes before releasing their toxic contents, unlike accidental cell death or necrosis. Second, excessive apoptosis may cause diseases [54] (Fig. 8.3). There are two major signaling pathways of apoptosis. The extrinsic or receptor-mediated apoptosis is triggered by the binding of certain ligands such as FasL or the tumor necrosis factor (TNF) to cell death receptors on the surface [53, 55, 56]. Most cells in the human body have two receptors for TNF: TNF-R1 and TNF-R2. The binding of TNF to TNF-R1 has been shown to initiate the pathway that leads to caspase activation via the intermediate membrane proteins TNF receptor-associated death domain (TRADD) and Fas-associated death domain protein (FADD) [56]. The other receptor is Fas (CD95). When its ligand, FasL, binds to Fas, the intracellular death domain of Fas is in its turn combined with FADD. Once assembled, FADD leads to caspase-8 activation, which in its turn triggers the caspase cascade [43, 55]. The intrinsic pathway is triggered by a variety of stimuli (i.e., drugs, radiation, infectious agents and ROS, nitric oxide, high levels of calcium, as those induced by prolonged cell hypoxia, or the conformation shift of the cytosolic protein Bax) and involves the formation and

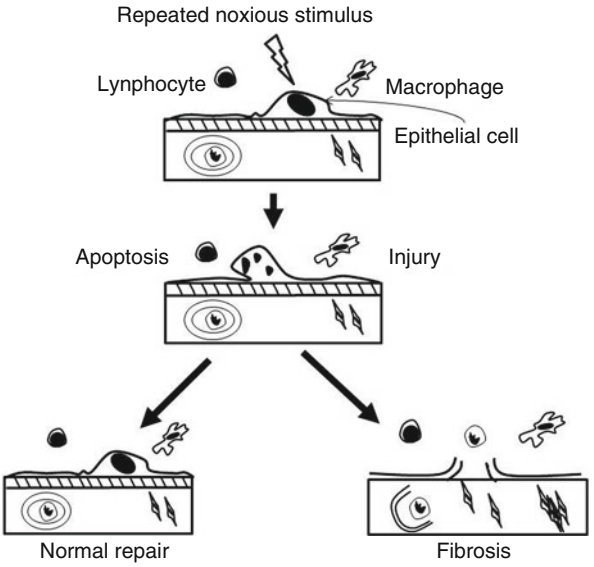

Fig. 8.3 Fibrotic mechanism. Depending on the degree of lung injury, the damaged tissue is normally repaired or to irrepairable damage and remodeling

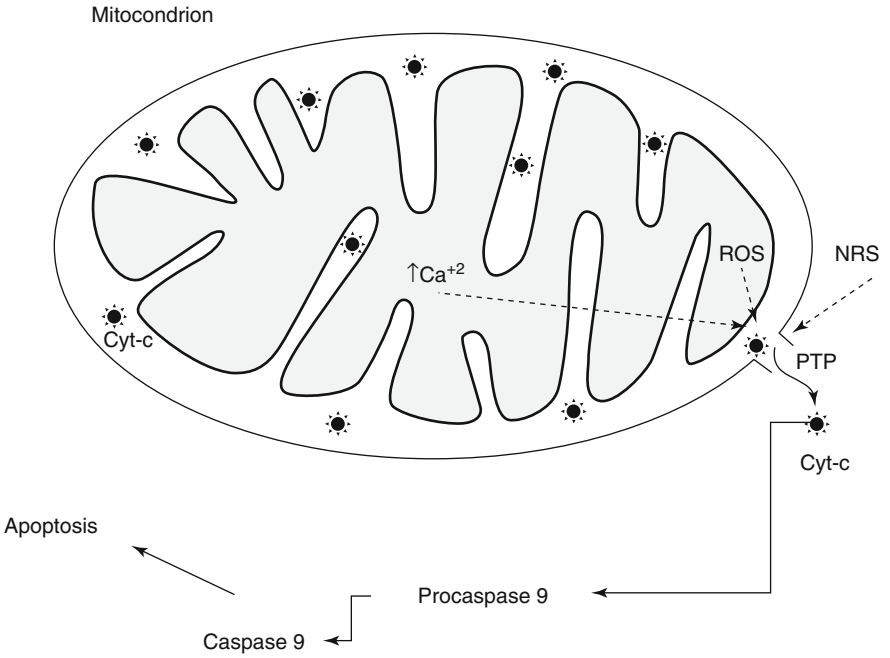

Fig. 8.4 Intrinsic mechanism of apoptosis. The intrinsic mechanism of apoptosis starts by the formation of the permeability transition pore (*PTP*) in response to several stimuli (ROS, NRS, high calcium levels, and radiation). The high-conductance pore allows the release of cytochrome *c* and other facts that initiate the caspase cascade by activating procaspase-9 into caspase-9. *ROS* reactive oxygen species, *NRS* nitrosative reactive species, *Cyt-c* cytochrome *c*

opening in a large conductance of the mitochondrial PTP, a nonspecific multiconductance channel of the outer membrane [43] (Fig. 8.4). After cytochrome c is released into the cytosol from the mitochondria, it binds to Apaf1 and ATP, which then activate caspase-9 [43, 55]. In this section we will deal with the information on intrinsic (i.e., mediated by mitochondria) apoptosis in lung disease.

Acute Lung Injury

Apoptosis of type I alveolar epithelial cells and endothelial cells are observed in acute respiratory distress syndrome (ARDS) [57], and the number of epithelial cell apoptosis is associated with the prognosis of patients with diffuse alveolar damage [58, 59]. The expression of Bax protein is upregulated in alveolar epithelial cells in acute lung injury [58, 59], and lipopolysaccharide, an important factor in acute lung injury, induces apoptosis of endothelial cells of systemic organs in mice [60]. Cytokines with antiapoptotic properties such as IL-6, IL-11, IL-15, and granulocyte-macrophage colony-stimulating factor attenuate hyperoxic lung injury, and this protection is associated with a marked decrease of apoptosis [61–64]. These findings suggest that apoptosis plays a major role in the development of acute lung injury in mice and ARDS in human.

Interstitial Lung Diseases

Alveolar epithelial damage is an important initial event in idiopathic pulmonary fibrosis (IPF) which includes an augmented epithelial cell apoptosis [65–67]. Both the Fas/FasL- and mitochondria-mediated apoptotic pathways are activated in IPF [68]; however, some evidence suggests that Fas-mediated pathway is more relevant [69].

Bronchial Asthma

The loss of columnar epithelial cells is a characteristic feature of asthma. Asthmatic bronchial epithelium is susceptible to H_2O_2-induced apoptosis [70]. Such susceptibility may contribute to the rising trends in asthma associated with air pollution and diets low in antioxidants [70]. In severe asthma, there is a greater level of apoptotic activity and increased cellular proliferation in the airway epithelium [71]. Since the remodeling processes of bronchial epithelium are associated with various molecules which are involved in epithelial cell apoptosis, treatments targeting epithelial cell apoptosis may be effective in these patients.

Chronic Obstructive Pulmonary Disease

Increased numbers of apoptotic alveolar, bronchiolar, and endothelial cells are frequently observed in lung tissues from patients with COPD [53]. Apoptosis has been associated with the initial injury and defective repair in COPD and the destruction and loss of alveolar structures characteristic of pulmonary emphysema [72, 73]. Since apoptosis is likely to be involved in not only the destructive phase but also remodeling process, regulation of apoptosis may become an effective treatment against COPD.

Cigarette Smoke

Cigarette smoke induces epithelial cell apoptosis [74]. Moreover, it impairs the removal of apoptotic cells [75], and the failure to remove is thought to be also important as a contributor to COPD [76]. CS also causes mitochondrial dysfunction by blocking mitochondrial respiratory chain and decreasing ATP generation which leads to cellular death (apparently necrosis rather than apoptosis) [77]. The heme oxygenase-1 (HO-1) mRNA expression was elevated in the lungs of mice chronically exposed to cigarette smoke. The mitochondrial localization of HO-1 in bronchiolar epithelial cells was confirmed using electron microscopy. Overexpression of HO-1 inhibited cigarette smoke extract induced [78].

Skeletal Muscle Dysfunction of COPD

There is compelling evidence showing the involvement of mitochondria in the muscle dysfunction (SMD) associated to COPD. Typically the citric acid cycle (Krebs cycle) enzyme activity (such as citrate synthase, SDH) and β-hydroxyacyl-CoA dehydrogenase (an enzyme involved in β-oxidation of fatty acids) are lower in patients with COPD as compared with control individuals [79–82]. Conversely, cytoplasmic glycolytic enzyme activities are elevated [79–82]. Patients with mild or moderate COPD usually show a reduced "vastus lateralis" state 3 mitochondrial oxygen consumption, acceptor control ratio, and ATP production [83–86]; however, when normalized by citrate synthase (CS) activity, a measure of mitochondrial density, differences between COPD and normal individuals in mitochondrial oxygen consumption, and ATP production have been found small [84] or nil [85]. In keeping with these findings, measurement of mitochondrial density has been found lower in COPD than in age-matched, healthy controls [87, 88]. The reduction of mitochondrial density and oxidative fiber loss is the typical physiological response of muscle [89, 90]. Moreover, supporting the role of deconditioning, peripheral muscle oxidative capacity has been shown to improve with training [81, 91, 92] although not at the same levels observed in healthy subjects [93].

Conversely to what occurs in the lower limb muscles, the activities of the oxidative enzymes such as CS, SDH, and δ-hydroxyacyl-CoA dehydrogenase are elevated in the diaphragm of patients with severe COPD [94–96], whereas the activities of the glycolytic enzymes are decreased [97]. Enhanced mitochondrial state 3 respiration has also been reported in the diaphragm of very severe COPD patients, and significant correlations were found between the maximal oxidative capacity and patients' pulmonary indexes of obstruction [98]. These data indicate that the changes in oxidative capacity and mitochondrial function in the diaphragm are in the opposite direction than in the "vastus lateralis" and they appear to become more marked with the progression of the obstruction. This likely reflects a training effect of the muscles [94, 95, 97].

Excessive ROS production is another feature of SMD in COPD. The rates of generation of ROS and other free radicals are elevated in patients with COPD during exercise [99, 100], and mitochondria have been shown to be major contributors [30, 84]. The sources of these oxygen free radicals are complexes I and III, but only those ROS generated by complex III appear to be able to reach the cytoplasm [30, 101]. The oxidative stress associated to excessive ROS production affects membrane lipids, proteins inside and outside the mitochondria, and mitochondrial DNA [88, 101–103] impairing mitochondrial oxidative function by jeopardizing the integrity of the electron transport chain and uncoupling oxidative phosphorylation by decreasing the transmembrane potential [43, 104, 105]. Protein oxidation may modify the structure or chemical properties of the proteins affected [102, 106] and cause a decline in protein function or even complete protein unfolding in muscle cells [101, 104]. Among the proteins affected are the myofibrillar muscle protein that becomes more susceptible to proteases [107], thus contributing to the dysfunction of the muscle.

Conclusions

The mitochondrion of the eukaryotic cells is not only the powerhouse of the cell that generates energy but is also involved in several important cellular functions including cell signaling and death. The mitochondrion also acts as the oxygen sensor especially during hypoxia and signals the information toward the generation of ROS that act as mediators of cell signaling. This hypoxia-induced ROS signaling during hypoxia is believed to be responsible for hypoxic vasoconstriction, activation of HIF, and internalization of the sodium-potassium pump of the alveolar epithelium. In cancer cells, mitochondrial DNA mutations lead to compromised oxidative metabolism and decreased susceptibility to apoptosis that are crucial for survival of the cancer cell. One of the two major signaling pathways of apoptosis involves the mitochondria. Apoptosis is an important player in several lung diseases that contributes to the failure of clearance of unwanted cells and prolonged inflammation and thus is involved in the pathogenesis of the lung diseases. The skeletal muscle dysfunction associated with COPD involves the loss of oxidative capacity, mainly due to the loss of mitochondrial density.

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Chapter 9
Age-Specific Difference in Pulmonary Cellular Injury and Mitochondrial Damage

Katherine L. Tuggle and Michelle V. Fanucchi

Abstract The impact of lung-targeted toxicants on the respiratory system of developing and aging animals is not well defined, let alone the role that mitochondria may play in the cascade of factors involved in cellular injury. Environmentally induced pulmonary cell injury is often due to exposure to bioactivated toxicants or oxidant gases. The major defense mechanisms against these insults are the antioxidant and xenobiotic-metabolizing enzymes. The age-specific expression of these enzymes and their roles in protecting the lung from specific toxicants is discussed as well as the interrelationship with mitochondrial function. A review of the literature reveals evidence of the mitochondrial involvement in cellular injury during lung development and aging. Whether the alterations of mitochondria are critical early steps in lung injury or an outcome of a cascade of processes during lung development and aging is not clear at this time.

Keywords Lung development • Lung aging • Mitochondria • mtDNA • Antioxidant • Tobacco smoke • Ozone • Hyperoxia

Introduction

This chapter reviews the literature on cellular injury during pulmonary development and aging and its relationship with mitochondrial health and function. The relationship between mitochondrial function and age-associated lung injury has been

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recently evaluated. However, mitochondrial injury has been reported as an ancillary endpoint in earlier toxicological studies. This chapter focuses on the information extracted from the discrete results that were not necessarily highlighted in abstracts or publication titles.

Mitochondria

Mitochondria are involved in numerous important cellular processes including energy production, embryonic development, aging, and cell death [1]. Recently, mitochondria have also been recognized as important targets of toxic agents [2–4] (Table 9.1). Some of the primary mechanisms of mitochondrial toxicity are inhibition of the electron transport chain, uncoupling of oxidative phosphorylation, damage to mtDNA, and inhibition of mtDNA synthesis (Fig. 9.1). With a wide range of biological processes that occur within this highly specialized organelle, disruption of any of these processes has the potential to alter the bioenergetic status of the cell, leading to altered cellular homeostasis and cell death. Classically, mitochondria have been associated with their role in energy production, but they are also the predominant generators of intracellular reactive oxygen species (ROS), specifically superoxide (O_2^-) [5, 6]. In addition to direct actions of toxic agents on mitochondrial components, including the electron transport chain proteins, many compounds are also capable of generating or increasing mitochondrial ROS in the matrix. In healthy mitochondria, 1–2 % of oxygen forms reactive species, including free radicals (such as superoxide) and peroxides [5, 7]. However, during toxic insults and in diseased mitochondria, ROS production can exceed antioxidant capacity, increasing the susceptibility of mitochondria to ROS-induced damage of DNA, lipids, and proteins [8, 9]. Increased mitochondrial damage can cause mitochondrial dysfunction and activate both apoptotic and necrotic cell death pathways. Mitochondria also regulate the

Table 9.1 Mitochondrial targets of toxic compounds

Target	Action/result
Complex I	↓ Oxidative phosphorylation → ↓ ATP production ↑ ROS
Complex III	↓ Oxidative phosphorylation → ↓ ATP production ↑ ROS
Complex IV	↓ Oxidative phosphorylation → ↓ ATP production ↑ ROS
Complex V (ATP synthase)	↓ ATP production ↑ ROS
Membrane uncoupling	↓ Membrane potential ($\Delta\Psi$) → ↓ ATP production
mtDNA	Defective protein synthesis → ↓ oxidative phosphorylation → mitochondrial dysfunction
β-Oxidation	↑ Oxidative stress
Permeability transition pore	↑ Mitochondrial swelling → cytochrome C release → cell death (necrosis/apoptosis)

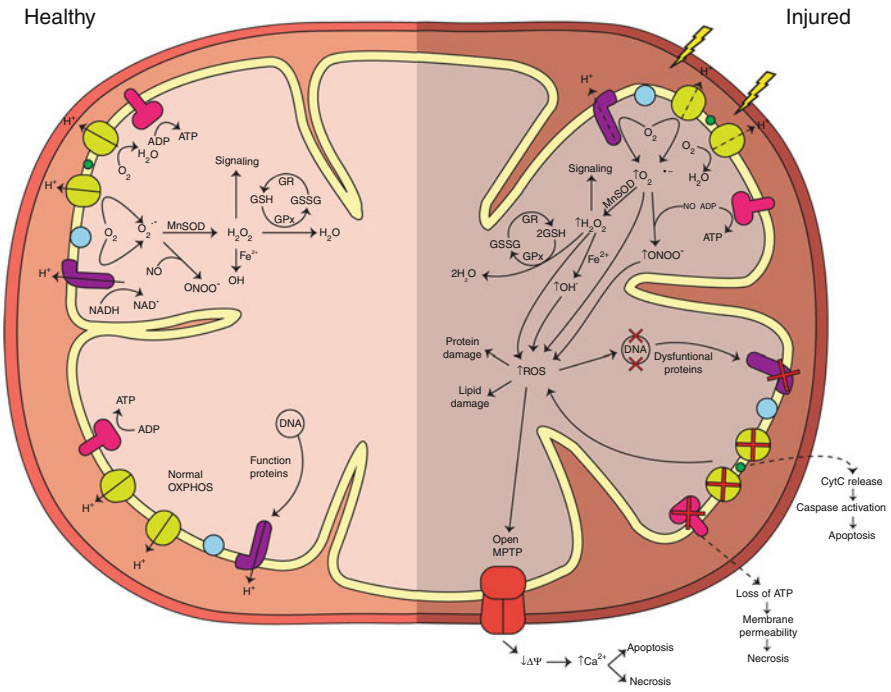

Fig. 9.1 Diagram showing the relationship between ROS generation, ATP production, and cell death pathways in a healthy mitochondrion (*left side*) and an injured mitochondrion (*right side*).

release of proapoptotic mediators, including cytochrome *c* and apoptosis-inducing factor, in response to both extrinsic (cell surface mediated) and intrinsic signals [10]. After release from the mitochondria, cytochrome *c* will complex with apoptotic protease activating factor-1 (Apaf-1) to activate caspase-9 and form the apoptosome. The apoptosome activates caspase-3 and other downstream executioner caspases to advance the apoptotic pathway. Apoptosis is an ATP-dependent process [11]. In contrast to the energy-consuming apoptosis pathway, necrotic cell death results when ATP levels are depleted [12, 13]. As the essential organelle involved in balancing between life (ATP production) and death (necrotic/apoptotic pathways), mitochondria play a critical role in cellular responses to toxic insults.

Since mitochondria are constantly exposed to low levels of ROS, they have adapted by developing an antioxidant response system to reduce the oxidant burden in the mitochondria and preserve mitochondrial integrity and function. Mitochondria are equipped with high concentrations of glutathione (GSH), superoxide dismutase, glutathione peroxidase, and other antioxidant enzymes to remove potentially harmful oxidants; however, even in mature mitochondria, increased production of ROS can quickly overwhelm these systems [14]. The expression of many of these systems changes over the course of postnatal lung development, potentially endangering children vulnerable to increased damage due to pulmonary damage during this period (Table 9.2). The expression of those systems are again changed with

Table 9.2 Age-related factors that alter susceptibility to pulmonary cellular injury

Immature	Aged
Incomplete development of detoxification enzyme systems	Damage to enzyme systems
Increased cellular proliferation	Reduced repair/regeneration capacity
Increased breathing frequency	Cumulative oxidative damage to DNA, proteins, and lipids
Increased lung surface area/body weight	Decreased immune function
Immune system is not fully developed	Reduced mitochondria turnover
Incomplete airway and alveolar development	Reduced airway clearance mechanisms
Morphological changes in mitochondrial structure	Increased caspase activity

advancing age due to cumulative lifetime protein and DNA damage that can alter activity or decrease protein expression, which can result from the failure of multiple systems. Animal studies have shown that supplementation with ascorbic acid and other antioxidants are capable of protecting the lung against age-related oxidative mitochondrial DNA (mtDNA) damage [9].

During postnatal lung development, mitochondrial volume increases and morphological alterations occur primarily in nonciliated epithelial cells and type II alveolar cells, two proliferating cell populations in the airways and alveoli [15, 16]. In the rabbit and rat, mitochondrial volume increase during both pre- and postnatal development in the nonciliated bronchiolar cells, including Clara cells [15, 17]. In prenatal rabbits, less than 15 % of the volume in Clara cells is mitochondria; however, by 4 weeks postnatal, Clara cells resemble that of older animals, indicating that mitochondria undergo a shift in abundance during differentiation [18]. In the rat, during postnatal development, mitochondria change from a single spherical organelle to a complex branched structure in type II alveolar cells [16]. Changes in mitochondrial size and intracellular distribution during cellular differentiation may alter cellular responses to injury and alter the ability of the cell to recover from toxic insults. In contrast to the changes that occur during development, advanced age also alters mitochondrial dynamics. The mitochondrial theory of advanced aging has been widely accepted since its introduction in the 1970s and is believed to be the driving force behind cellular aging in the body [19, 20]. Age-related changes in basic mitochondrial functions have been reported, including decreased respiratory control ratios (RCR), changes in mitochondrial membrane permeability ($\Delta\Psi$), and activities of the electron transport chain complexes (I, III, and IV), which are believed to be the result of cumulative oxidative damage [21].

Mitochondrial DNA (mtDNA)

Mitochondrial DNA (mtDNA) is a double-stranded circular DNA that encodes the mitochondrial genome and resides in the mitochondrial matrix near the electron transport chain. In humans, the mtDNA genome has 16,569 bp and contains 13

protein-coding genes, most of which are electron transport chain (ETC) proteins. Other animals, including mice, rats, and primates, have slight variations in the size of the DNA (16.3–16.5 kb) [22]. mtDNA lack introns, so mutations in mtDNA are likely to affect the coding regions which may result in the production of dysfunctional proteins [23]. In contrast to nuclear DNA, mtDNA lack protective histones and therefore are at increased risk for oxidative damage from the ETC, the primary source of ROS in the mitochondria [24]. With this proximity to the oxidant producing ETC, mtDNA is prone to oxidative damage that can alter protein synthesis of critical proteins in the ETC and ultimately decrease the ability of cell to produce energy. To protect the mtDNA, a combination of DNA repair mechanisms and degradation of irreparable mtDNA are used to maintain integrity [25]. Unlike damaged nuclear DNA, replication of damaged mtDNA is not blocked, allowing defective or nonfunctional proteins to be synthesized and inserted into the ETC [26]. Oxidative lesions of mtDNA also accumulate with age in rodents [27] and humans [28], with levels of mtDNA damage being significantly greater than those found in nuclear DNA [9]. Under normal physiologic conditions, it is estimated that DNA damage ranges from 1 modification for every 130,000 bases of nuclear DNA to 1 in 8,000 bases for mtDNA [29]. mtDNA damage is also correlated with oxidant-mediated cell death [30, 31]. mtDNA deletions in conjunction with membrane lipid peroxidation may also play a key role in mitochondrial dysfunction due to aging [27].

In the mouse lung, mtDNA content increases during the first 2 months of postnatal development, drops slightly around 5 months, and increases again by 15 months of age, showing that the cyclic increase of mtDNA levels closely correlates with two periods of increased susceptibility to oxidative stress and injury, during early life and advanced age [32]. In humans, mtDNA content increases by 2.6-fold in lung tissue from patients over 80 as compared to those less than 20 [28]. Tissues from aging and senescent rats have been shown to contain increased mtDNA as compared to younger age groups [27]. mtDNA lesions increase in postmitotic tissue as compared to mitotic tissues in humans; however, one study using Fisher 344 rats reveals the opposite effect [33].

Mitochondrial Dysfunction

Mitochondrial dysfunction occurs when the mitochondrial function is impaired resulting in reduced energy production. When electron transfer between oxidoreductase complexes becomes impaired, the matrix becomes a more electron-rich environment, which increases autoxidation, increases ROS generation, and decreases respiration [34, 35]. Provided that energy production is essential for cell survival, any dysfunction that occurs in the mitochondria has the potential to affect cellular homeostasis and shift toward cell death.

In the mitochondria, two common measures of respiratory function are state 3 and state 4 respiration [36]. State 3 respiration refers to the state of actively respiring mitochondria in which ADP is converted to ATP. In contrast, state 4 respiration

is the slower rate of respiration that occurs after ADP has been depleted. Mitochondria are believed to function between state 3 and state 4 in vivo. The RCR is a ratio of state 3 to state 4 respiration which serves as an index of tightness of coupling between respiration and phosphorylation. The relationship between ATP synthesis (during the respiratory burst of state 3 respiration) and oxygen consumption is determined by the P:O ratio [37].

In the lung mitochondria of senescence-accelerated mice, advanced age results in reduced activity of the electron transport chain complexes (I–IV) and ATP production [38]. In Fisher 344 rats, using succinate as a substrate, state 3 respiration, uncoupled state respiration, and respiratory control ratio (RCR) are all significantly decreased in aged rats as compared to adults [39]. Following exposure to 3 ppm ozone for 8 h, respiration rates for state 3, state 4, and the uncoupled state, as well as the RCR and ADP/O ratios, decrease similarly in both adult and aged rats [39]. This same study also compares isolated heart and lung mitochondria and shows a greater decrease in metabolism in lung mitochondria. This may be attributed to proximity of ozone-derived products and their ability to interact with lung mitochondria. Ozone also induces changes in the presence of NAD- and FAD-linked substrates that may indicate alterations in the inner mitochondrial membrane. Mitochondria may be a subcellular organelle target for ozone interactions and that alteration of energy metabolism may contribute significantly to the overall damage caused by this oxidant. Impaired energy production in the aged mitochondria may contribute to the age-related physiological consequences and accelerated alterations during chemical stress [39]. Compared to 5-month-old mice, 10-month-old mice show decreased activity in complexes 1, 2, and 4 in both male and female mice, while complex 3 is depressed only in male mice [38]. Additionally, ATP production and ATP/ADP ratios are depressed in the 10-month-old mice, indicating that mitochondria in the aged mice are not as efficient in production of energy as the adult mice [38]. State 3 and state 4 O_2 consumption is significantly higher in immature rats compared to both adult and aged rats; however, O_2 consumption is not significantly different between adult and aged groups. RCR remains constant across all age groups. H_2O_2/O_2 consumption in state 4 is significantly lower in the young and aged rats as compared to the same in adults [40].

General Considerations for Lung Development and Aging

The adult lung is comprised of many morphologically distinct cell phenotypes organized into a highly branched series of tissues surrounding the air passages. Each developmental stage of the lung is associated with the transformation from a simple tubular structure into a highly complex organ system that is potentially susceptible to modification by toxic agents. As a result, several aspects of the developmental process should be considered when evaluating the potential toxicity of a compound. First, it is important to remember that lung development is a multi-event process which is not restricted to prenatal life. Although the development of the

tracheobronchial bud begins early in gestation, a large amount of lung growth and therefore cellular differentiation occurs during the postnatal period. Second, only a restricted number of maturational events must be complete at birth for successful postpartum survival of an organism. Third, there are three general categories of events that occur throughout the pre- and postnatal development periods – branching morphogenesis, overall growth, and cellular differentiation – that may alter the toxicity of a compound in the lung. The process of branching morphogenesis begins with the formation of the tracheobronchial bud. This branching continues until there is an extensive airway tree of about 22 generations in humans. The formation of vascular trees, arterial and venous, and capillary beds associated with both the conducting airways and the gas-exchange region occurs at the same time as the airway tree. The highly complex structure of the gas-exchange area also requires an extensive period of branching morphogenesis to form alveolar saccules and ducts, most of which occurs during the postnatal period. Formation of the interalveolar septa, a process termed "alveolarization," begins during late gestation and continues through the first 2 years of life in humans [41, 42]. During the alveolarization period in rats, type II cells undergo a series of morphological changes, including a shift from single spherical mitochondria to more complex branched structures [16]. The overall size, or volume, of the lungs and trachea increases in a steady progression throughout pre- and postnatal life until body size ceases to increase. The cells of the airway tree, matrix, and interstitium differentiate at varying rates. Fourth, all of these developmental events occur in combination with a steady increase in total cell mass, primarily through cellular proliferation.

Even though federal agencies have identified children as a susceptible population, the impact of toxicants during developmental of the lower respiratory tract is not well understood. One reason is that the mechanisms regulating the complex series of morphogenetic and events of differentiation are only now beginning to be identified. Some of the regulatory factors, such as hormones and cytokines, have been recognized, but epithelial-matrix interactions and cell-cell (epithelial-epithelial or epithelial-mesenchymal) interactions are only starting to become evident. Toxicants can modify developmental events in many ways. For example, exposure to a toxicant can result in a poorly functioning, or inadequate, gas-exchange system. Toxicants can also induce a partially differentiated cell population to develop phenotypes not found in uninjured lung or alter the expression of phenotypes present so they are more or less resistant to injury.

Once development and growth is finished, we must then consider the issues associated with the aging of the lung. With increasing age, numerous physiological changes occur in the lung, including reduced ventilatory control, respiratory mechanics, and gas exchange [43]. In the aged lung, respiratory muscle strength decreases resulting in impaired ability to cough, which is an important mechanism for airway clearance [44]. Additionally, aging alters deposition, retention, and clearance of particles [44]. Alterations are noted in structural components of the airway and alveolar wall, indicating impaired repair mechanisms, many of which are associated with age-related lung diseases [44, 45]. One of the driving forces behind aging is believed to be cellular senescence. Senescence is a state in which cellular

proliferation is arrested and cells also become resistant to apoptosis [46]. Typical responses to DNA damage enable the cell to repair the damage when possible; however, if the cell is unable to do so, it will respond by arresting cell-cycle progression [47]. Senescent cells have been shown to increase expression of genes, including extracellular matrix degrading enzymes, inflammatory cytokines, and growth factors [46]. These factors disrupt normal tissue structure and function and also affect non-senescent cells by causing abnormal responses to stimuli [46]. In rat lung and liver, advanced age is associated with increased activation of executioner caspase-3, caspase-6, and caspase-7 and progression of apoptosis [48]. Additionally, advanced age also increases activity of caspase-2 and caspase-9, both of which are involved in mitochondria-mediated apoptosis in liver suggesting that as cells age, mitochondria-centric cell death pathways may be more active.

Cellular Antioxidant and Detoxification Systems

The mature lung has well-developed antioxidant and xenobiotic metabolism systems to detoxify endogenous and exogenous compounds that have the potential to cause cellular injury. Many of these detoxifying enzyme systems are confined to subcellular compartments (i.e., cytoplasm, mitochondria, endoplasmic reticulum); however, properly functioning systems in all compartments are essential for protecting the cell as a whole. The following sections describe the development of these systems during early life and the loss of activity with advanced aging that may alter susceptibility to cellular injury in these age groups.

Age-Specific Expression of Antioxidant Enzyme Systems

An obvious role of antioxidants in the lung is protecting the lung from oxidative stress. This level of protection is critical at birth, when the lung switches from a relatively hypoxic state to a relatively hyperoxic state. Additionally, as the lung ages, oxidant burden continues to increase. The antioxidant enzymes [superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx)] and nonenzymatic antioxidants (glutathione, GSH) also have an important role in modulating cellular interaction with environmental toxicants such as ozone and nitrogen dioxide. During late gestation, changes in the fetal lung include the development of the antioxidant enzymes, including SOD, catalase, and GPx. These enzymes play an important role in the detoxification of highly reactive oxygen species that are produced during normal aerobic cellular respiration as well as during oxidant injury. In general, the pulmonary antioxidant enzyme system develops during the last 10–15 % of gestation in humans [49] as well as in many laboratory animal models including rats, hamsters, guinea pigs, rabbits, and lambs [50–58].

Superoxide Dismutase (SOD)

The antioxidant enzyme SOD rapidly catalyzes the conversion of the superoxide anion to hydrogen peroxide and oxygen. There are three isoforms of SOD: copper-zinc SOD (Cu/ZnSOD), manganese SOD (MnSOD), and extracellular SOD (EcSOD), all of which are found in the lung. MnSOD, an inducible form of superoxide dismutase, is predominantly located in the mitochondria [59, 60], while the noninducible Cu/ZnSOD is located in the cytosol [61]. EcSOD is localized in the extracellular matrix, vessel walls, and extracellular fluid of the lung [62, 63]. EcSOD is thought to play a role in modulating nitric oxide concentrations by regulating the amount of superoxide anion available to react with it [64], but very little is known about this form in the developing lung [65].

In most animal models, SOD activity increases throughout the pre- and postnatal periods; however, there are some species differences (Fig. 9.2). In rats, pulmonary Cu/ZnSOD activity and total enzyme content peak during the late gestational period (last 10–15 % of gestation) and activity peaks again early in the postnatal period, finally reaching adult levels at 4 weeks [54, 56, 67, 68, 72]. Cu/ZnSOD activity is elevated in young rats compared to adult and aged rats (40). Temporal expression of

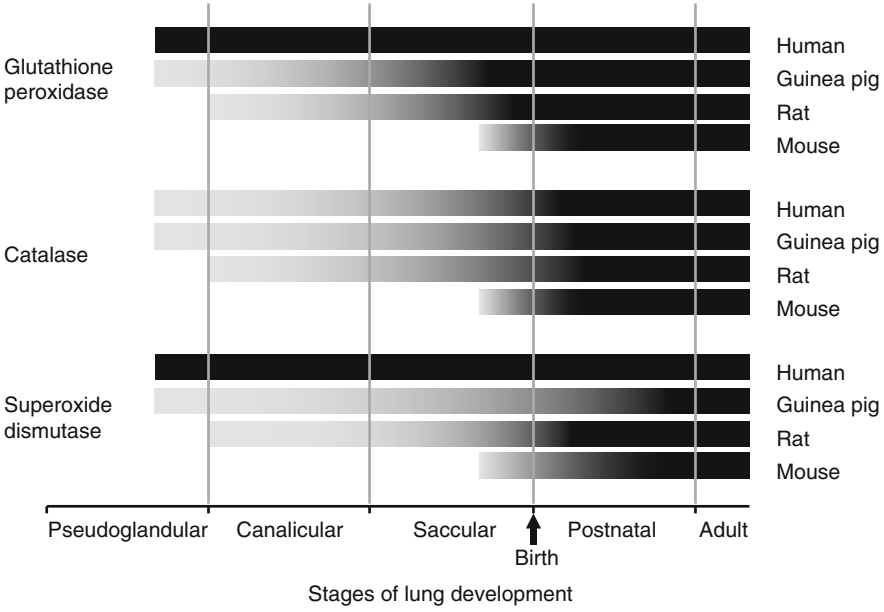

Fig. 9.2 Species-dependent expression of antioxidant enzyme protein and/or activity during stages of lung development. *Gray portion of bars* represent transitional expression, *black portions of bars* represent mature expression for human [49, 66], guinea pig [55], rat [50, 54, 67–70], and mouse [291] (Reprinted with permission from Fanucchi [71])

mRNA does not correlate with the activity levels [68, 73]. In contrast, pulmonary MnSOD activity and mRNA content remain steady throughout late gestation and the early postnatal period in the rat [51, 72, 73], even though total enzyme content increases 6.9-fold [54]. In the mouse, Cu/ZnSOD mRNA expression peaks at 15-day gestation, is low during the later stages of fetal lung development, and peaks again at birth [73, 74]. In rabbits, pulmonary Cu/ZnSOD and MnSOD activities also peak in late gestation and in the postnatal period [55]. In the rabbit, EcSOD expression is low and contained within the intracellular compartment during the fetal period. Expression increases and shifts to the extracellular compartment with increasing age [65]. In the developing rat, EcSOD protein expression and activity increase from 2 days of age until adulthood [75]. In contrast to experimental animals, human Cu/ZnSOD and MnSOD activity remains constant during fetal and neonatal lung development, and no surge in expression is seen [49, 66].

In the aged lung, SOD activity and protein expression results have been across the board. In one study, SOD activity (total SOD, Cu/ZnSOD, and MnSOD) is not altered in the lungs of aged rats [76]. Another study found that MnSOD and Cu/ZnSOD activities increase in aged rats (26 months) as compared to young (4 months old) rats; however, mRNA expression is not altered by age [77]. While some of the results are mixed, majority of the literature has shown that SOD protein expression and activity decrease in the lung tissue of aged rats [78–81].

Glutathione Peroxidase (GPx)

GPx is a cytosolic enzyme that catalyzes the reduction of hydrogen peroxide to water and oxygen as well as catalyzing the reduction of peroxide radicals to alcohols and oxygen [52]. The oxidation of GSH to GSSG provides energy for this catalysis, but the higher oxidation state can be toxic, because NADPH is consumed when GSSG is reduced back to GSH in cells.

As with SOD, GPx activity and total enzyme content increase during late gestation in two of the experimental animals studied: guinea pig and rat [54, 56, 72, 73] (Fig. 9.2). However, mRNA expression steadily decreases during fetal development and does not correlate with activity levels [51, 73]. GPx activity in postnatal rats is lower than adults, suggesting that GPx activity increases during the postnatal period [40]. In the mouse, however, both GPx activity and mRNA levels peak in late gestation and then activity steadily decreases throughout the postnatal period [20, 74]. In humans, GPx activity is lowest at 10–20 weeks of gestation, is highest during the early postnatal period (41–50 weeks postconception), and drops at 3–5 months of age [49, 82].

GPx mRNA expression decreases in the lungs of aged rats as compared to young adult rats [77]. In addition, GPx activity decreases in aged rats compared to young adult rats [40, 77–79, 81]. In senescence-accelerated mouse model, GPx activity has also been shown to decrease with age [38].

Catalase

Catalase, like GPx, accelerates the combination of two hydrogen peroxide molecules to produce water and oxygen. Catalase activity steadily increases during both the fetal and postnatal periods of lung development in humans [49] and in all experimental animals studied, including guinea pigs, rats, and mice [20, 54, 56, 72, 73] (Fig. 9.2). In contrast to GPx, the catalase mRNA expression during this time is consistent with the increase in activity [73]. In humans, catalase activity increases 3.5-fold between 10 weeks of gestation and 3 months of postnatal age, unlike SOD and GPx, which remain constant throughout human lung development [49]. The increase in catalase activity during the late gestational period has been suggested by McElroy and colleagues to be linked to maturation of the surfactant system and has been correlated temporally to increases in lung dipalmitoylphosphatidylcholine (DPPC) content [49].

The effects of advanced aging on catalase activity are mixed. In rats, mRNA expression is significantly lower in the aged group as compared to the young rats [77]. Catalase activity has been shown to decrease in aged 129/ReJ mice [83] and rats [78, 79, 81]. However, other studies have shown catalase activity unaffected by advanced age in C57BL/6 K mice [83] or rats [40, 77]. In one study, catalase activity is found to be increased in the lungs of aged BALB/c mice [83].

Glutathione

Glutathione (γ-glutamylcysteinylglycine, GSH), a tripeptide, acts as an antioxidant by directly scavenging free radicals through the donation of a hydrogen atom. In the lung, GSH is found in both intra- and extracellular compartments. In the cell, 85–90 % of the GSH resides in the cytosol, 10–15 % localized in the mitochondria, and small percentages in the endoplasmic reticulum and nucleus [84]. The synthesis of GSH is homeostatically controlled, both inside the cell and outside [85–88]. GSH is not taken up by pulmonary cells in its intact form. It is synthesized and utilized through the γ-glutamyl cycle, a multistep, ATP-dependent process. Very simplistically, it begins with the cleavage of extracellular GSH and other glutamate-containing peptides by γ-glutamyl transpeptidase, a membrane-bound enzyme. The resulting amino acids can then be moved by specific transporters into the intracellular environment [89]. The rate-limiting step in the synthesis of glutathione, however, is the formation of γ-glutamylcysteine by γ-glutamylcysteine synthetase (γ-GCS). The formation of GSH is complete when GSH synthetase catalyzes the addition of glycine to γ-glutamylcysteine to form γ-glutamylcysteinylglycine (GSH) [85]. Even though GSH plays an important role in protection against both oxidant and reactive metabolite injury, there is very little information available on glutathione levels or γ-glutamyl cycle enzyme activity during fetal and postnatal lung development.

Evidence from the few rodent studies available suggests that neonates have similar GSH levels as adults. In rats, pulmonary GSH levels are at mature levels right before birth, decrease 30 % at birth, and then increase back to mature levels between 10 and 15 days [90, 91]. The activities of two important enzymes of the γ-glutamyl cycle, γ-GCS and glutathione synthetase, are similar in the lungs of 1–2-week-old mice and adult mice [20]. As observed in the mice, there are no differences in the γ-GCS activity among the human fetal (15–19 gestational weeks), neonatal (26–42 gestational weeks), and adult lungs [69]. γ-Glutamyl transpeptidase mRNA is detectable by polymerase chain reaction in late fetal and early postnatal rat lung and is located in the type II cell [92]. However, pulmonary γ-glutamyl transpeptidase activity is very low in newborn rat, and only gradually increases to mature levels [92, 93]. In the late postnatal and adult rat lung, γ-glutamyl transpeptidase is found in higher abundance in Clara cells than in type II cells [92]. Pulmonary GSH synthetic activity is affected by several factors. One factor, maternal nutrition, has been shown to be very important in regulating the perinatal activity of GSH cycle enzymes. Pulmonary γ-GCS activity is lower in rat pups from dams that were fed low-protein diets than in pups from dams fed diets supplemented with casein [93]. Surgical procedures in newborn guinea pigs also affect GSH synthetic activity and result in increased pulmonary GSH content [94].

The effects of aging on tissue GSH levels rats are mixed. One study demonstrates that the status of reduced glutathione (GSH) is not different between the lungs of adult and aged rats [90]. However, multiple studies have shown a decrease of GSH in the GSH pool in the lung [79–81, 95, 96]. In the mouse lung, the ratio of GSSG (oxidized disulfide form of GSH) to reduced form of glutathione (GSH), a measure of oxidative capacity of the lung tissue, is increased with age suggesting the decreased ability to manage oxidative stress [96]. In the senescence-accelerated mouse, GSH/GSSG ratios decreases in the lung mitochondria with advanced age suggesting a reduced availability of GSH in this compartment [38]. The ability to synthesize and maintain reduced GSH decreases with age. GCL has also been shown to decline in the lung with advanced age. GSH reductase, however, is not affected by age [96]. In addition to alterations in tissue levels, GSH in the epithelial lining fluid decreases with age in humans [97] and mice [96]. The mitochondrial pool of GSH has been shown to be more oxidized with age, thereby reducing its protective effect in the mitochondria [98]. This change is greater in the mitochondria than in the whole cell [9].

Miscellaneous Antioxidants

Little information is available regarding the expression of other antioxidants during lung development. Ceruloplasmin, a free radical scavenger, is a major extracellular antioxidant in human lung epithelial fluid [99]. Ceruloplasmin mRNA has been detected in fetal lung of species as varied as mouse and baboon [100]. The mRNA first appears in baboon lung during the pseudoglandular stage (60-day gestation) in

bronchial epithelium. By the saccular stage of development (140-day gestation), ceruloplasmin mRNA is detected in all airway epithelium. In mice, ceruloplasmin mRNA is detected during the saccular stage and is expressed in epithelium of all airways. Peroxiredoxin, an antioxidant which reduces hydrogen peroxide to molecular oxygen has also been described in lungs of newborn baboons [101]. Both pulmonary peroxiredoxin mRNA and activity are expressed at low levels in fetal baboons and are upregulated by oxygen treatment. Urate and ascorbic acid (vitamin C) levels in bronchoalveolar lavage fluid have been described in preterm human infants [102]. Urate levels drop during the first 2 weeks of life, while ascorbate levels drop during the first week then increase during the second week of life. Ascorbic acid levels decrease again with advanced age [81, 103, 104]. Aging also reduces α-tocopherol levels in the lung [81].

Age-Specific Expression of Xenobiotic-Metabolizing Enzyme Systems

The interplay and balance between the xenobiotic-activating enzymes and xenobiotic-detoxifying enzyme systems have been shown to be critical factors in dictating the toxic response of the respiratory system to bioactivated compounds in adults [105–108]. This interplay is also important in perinatal animals during pulmonary morphogenetic and differentiation processes. Xenobiotic-metabolizing enzymes act on compounds to make them more water-soluble and increase their rate of elimination. Many lung-targeted toxicants, such as furans [109], chlorinated hydrocarbons [110, 111], aromatic hydrocarbons [108, 112], indoles [113–118], pyrrolizidine alkaloids [119], naphthalene [106, 120, 121], and butylated hydroxytoluene (BHT) [122–124], require bioactivation in order to exert their toxicity. The reactive metabolites of these bioactivated compounds are then detoxified by a number of pathways. Other compounds, such as organometallic [125] and amphiphilic agents [126], are toxic when introduced into the body and also require metabolic detoxification.

Cytochrome P450 Monooxygenases

The cytochrome P450 (CYP) monooxygenases are important enzymes in both bioactivation and detoxification of compounds. In adults, immunoreactive CYP enzymes have been detected in four pulmonary cell types: the nonciliated bronchiolar (Clara) cell, the type I and type II epithelial cells, the endothelial cell and the alveolar macrophage. The developmental expression of CYP monooxygenases is the most extensively studied of all the pulmonary xenobiotic-metabolizing enzymes. Even so, the expression of pulmonary CYP monooxygenases in the differentiating lung has been evaluated in only five species: rabbit, hamster, mouse, rat, and goat [120, 127–135]. Some of the CYP isozymes (*and representative substrates*) that

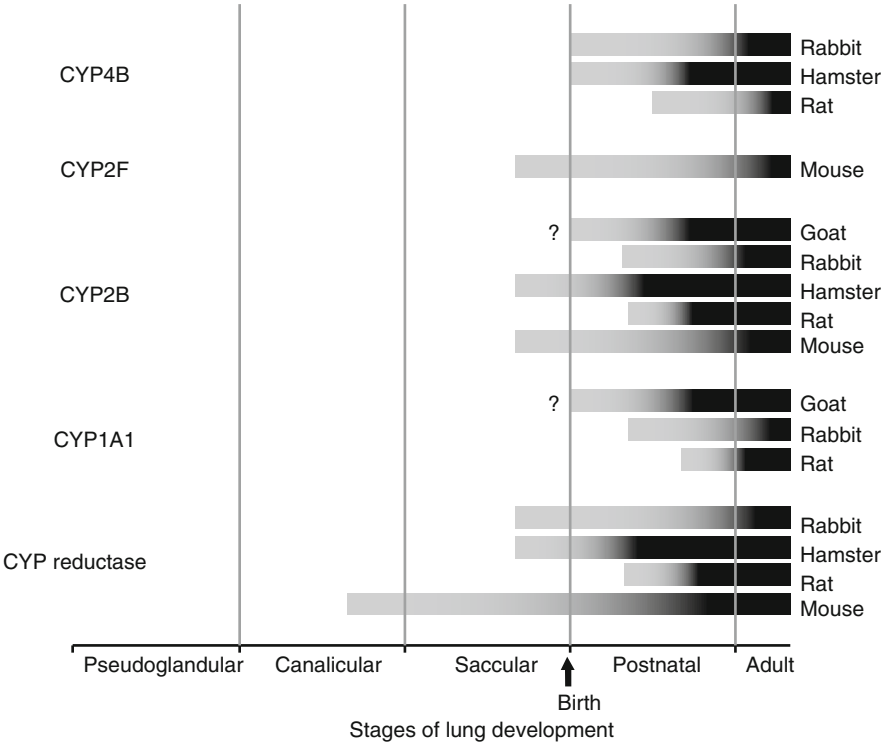

Fig. 9.3 Species-dependent expression of CYP monooxygenase protein and/or activity during stages of lung development. *Gray portion of bars* represent transitional expression, *black bar portions of bars* represent mature expression for goat [128], rabbit [136], hamster [135], rat [132, 137], and mouse [138]. *Question marks* (?) indicate lack of available information during the fetal period (Reprinted with permission from Fanucchi [71])

have been evaluated during lung development include CYP1A1 (*ethoxyresorufin*), CYP2B (*O,O,S-trimethylphosphorothioate*), CYP2F (*naphthalene*), and CYP4B (*2-aminofluorene*). In addition, NADPH CYP reductase, important for electron transport during the catalytic cycle of CYP monooxygenases, has also been evaluated. In general, the first detectable expression of CYP monooxygenases occurs after the development of the smooth endoplasmic reticulum (SER). Protein for NADPH CYP reductase is detected before the monooxygenase isozymes in all species evaluated. The youngest age at which the intracellular protein can be detected immunohistochemically varies substantially within the five species evaluated (Fig. 9.3). Isozymes CYP2B and CYP2F are expressed earliest: they are detectable in the late fetal stage in mice and hamsters [129, 135], in the early postnatal period of rabbits and rats [131, 136, 137, 139], and around 6 weeks of age in goats [127, 128]. CYP4B is detected 2–3 days of age later. Activity for these proteins is first detected approximately 2–3 days after the protein is immunologically detectable. The temporal and spatial distribution of immunoreactive pulmonary CYP protein has been described in detail for the rabbit [136]. Initially, immunoreactive protein is

detected only in the most apical border of a small percentage of the nonciliated cell population. During the period of time in which the amount of detectable protein increases, the distribution changes in two ways. First, an immunologically detectable protein is found in an increasing proportion of the nonciliated cells. Second, the distribution of detectable protein within an individual cell moves from the apex to the base until it is evenly distributed throughout the cell. The timeframe between when the protein is first detectable and when it reaches mature distribution and intensity also varies among species. Mature expression of CYP proteins can take up to 4 weeks in rabbits and mice [129, 136], 3 weeks in hamsters [135], and as little as 1 week in rats [132, 137]. Mature expression of the immunoreactive protein, however, does not necessarily indicate mature enzyme activity, especially in rats and rabbits [132, 137]. This suggests that the activity of these enzymes continues to increase after the protein density and organelle composition have reached adult levels. CYP3A is an important isozyme in the metabolism of many therapeutics and environmental pollutants as well as endogenous compounds that humans are exposed to daily [140] although most of the literature focuses on hepatic expression. Comparative studies of the postnatal pulmonary expression of isozyme CYP3A in the rat [141] indicate that this CYP activity is detectable in postnatal lung as well as adult lung. It is critical to understand the temporal and spatial development of CYP monooxygenase activity in order to be able to extrapolate among species.

Glutathione Transferase

It is essential to understand the temporal and spatial development of the detoxifying enzymes, including glutathione transferase, in order to appreciate the role they play in protecting the lung during development. Glutathione S-transferases (GSTs) are a group of dimeric enzymes present in multiple subcellular compartments, including the cytosol, mitochondria, and other membrane-bound organelles (including the endoplasmic reticulum and nucleus) [84] that catalyze the conjugation of GSH to electrophilic compounds. In human tissues, GSTs are grouped into seven classes [142–146]. Many of the classes have been described in adult lung, although only the expression of GST α, μ, and π has been evaluated in the developing lungs of mice [147, 148], rabbits [149], goats [127], guinea pigs [149], and humans [150–153] (Fig. 9.4). Unlike CYP monooxygenases, which are expressed in only four cell types in the lung, pulmonary GSTs are expressed in multiple cell types including both ciliated and nonciliated bronchiolar epithelial cells, alveolar epithelial cells, endothelial cells, and smooth muscle cells. The ubiquitous distribution of the transferase enzymes suggests a role in protecting cells from reactive intermediates from both exogenous and endogenous metabolism. In goats, rabbits, and guinea pigs, information regarding the expression of GSTs is available for only the postnatal period of lung development [127, 148]. Rabbits and mice at birth have pulmonary GST protein expression and activity levels similar to adults, while GST expression is not mature in goats until later in the postnatal period. In mice, the only laboratory animal in which GST expression has been described during fetal lung development,

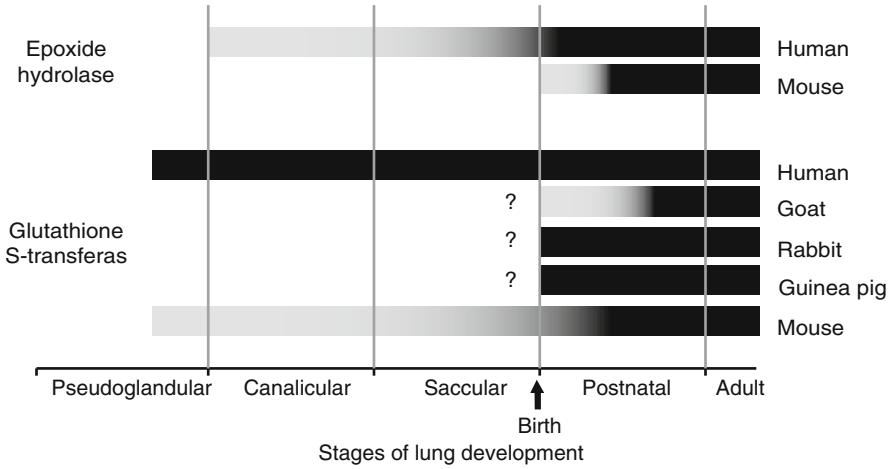

Fig. 9.4 Species-dependent expression of glutathione S-transferase (alpha, mu, and pi combined) and epoxide hydrolase (mEH and cEH combined) protein and/or activity during stages of lung development. *Gray portion of bars* represent transitional expression, and *black portions of bars* represent mature expression for goat [127], rabbit [149, 292, 293], guinea pig [149], mouse [148], and human [150–155, 294]. *Question marks* (?) indicate a lack of available information during the fetal period (Reprinted with permission from Fanucchi [71])

GST isozymes are present very early in development; however, the isozymes do not reach mature expression levels until early in the postnatal period [148]. In contrast to mice, human fetal lung has been shown to contain more overall GST activity than adult lung [153]. During human lung development, the total activity of pulmonary GST decreases by fivefold between 13 weeks gestation and birth and then remains constant [152]. Pulmonary GST (cytosolic) is significantly lower in weanlings than adult horses, suggesting that GST system continues to develop during the postnatal period [156]. Pulmonary GST activity is due to a combination of all the isozymes, and the contribution of each isozyme changes during development. In humans [150, 157, 158], GST1 (μ) increases as a percentage of total GST activity over development, from 5 % at 10 weeks of gestation (pseudoglandular stage of lung development) to 25 % at birth and 60 % in adult tissue. GST2 (α) contributes 50 % of the total activity consistently. GST3 (π) decreases as a percentage of total activity over development, from 50 % of the total GST activity at 10 weeks of gestation to 15 % at birth, and is almost undetectable in adult tissue. These activity data are supported by immunohistochemical data [151]. GST2 is expressed consistently throughout development: strongly in proximal airway epithelium and weakly in more distal airways in fetuses, newborns, and adults. GST3 decreases in expression during development. It is expressed strongly in all epithelial cells in early gestation, and then expression is lost in the distal airways by 24 weeks of gestation [151, 153, 159].

While much is known about GST activity in the developing lung, few studies have evaluated GST in the aged population. Studies in rats have differing results, finding either no alterations [78] or a twofold reduction in GST activity [81] with advanced age.

Epoxide Hydrolase

There are three general forms of epoxide hydrolase (EH) which all create 1,2-dihydrodiols from epoxides, although each form has different substrate specificities and tissue distribution [160]. The first form, microsomal EH (mEH), is involved in the conversion of cyclic epoxides and is found in high levels in the smooth endoplasmic reticulum (SER) and in lower levels in other membranous organelles. The second form, cytosolic EH (cEH), hydrates aliphatic epoxides and is found in the cytosol and peroxisomes. The third form, cholesterol EH, catalyzes the hydration of cholesterol epoxides and is located in the microsomal fraction. Substrate specificity may also differ immensely among species and tissues [160]. As with most metabolic enzymes, the highest levels of both mEH and cEH activity are found in the liver, and this is true in adults of a wide variety of species: rabbits [161], humans [161], rats [162], and mice [148, 163]. The ratio of cytosolic to microsomal EH activity, though, varies by species and by organ. In humans, cEH activity is higher than mEH activity in both the liver and the lung [154, 155, 159, 164]. In mice, while cEH activity is higher than mEH activity in the liver, the reverse is true in the lung [164]. EH activity also varies within the lung. EH activity is reported to be present in microdissected airways of dogs with the highest activity in distal airway generations and lower activities in proximal airway generations [165].

There are few studies on the development of epoxide hydrolase. In mice, the predominant pulmonary form of epoxide hydrolase is microsomal, with the cytosolic form found only in vascular smooth muscle [148]. Immunoreactive mEH protein is detectable in airway epithelial cells soon after birth and reaches mature density and distribution near the age of weaning (Fig. 9.4). However, specific activity for mEH in mouse lung is detectable at mature levels at 7-day postnatal age [148]. In contrast to the mouse, the predominant pulmonary form of epoxide hydrolase in humans is cytosolic and EH mRNA expression gradually increases to adult levels by 65 days postnatal [134]. Increases in fetal human pulmonary EH activity do not correlate with increases in mRNA expression [154]. Pulmonary soluble epoxide hydrolase did not vary with age in horses [156]. In mice, mEM activity decreases from 3 to 12 months of age followed by periods of increased and decreased activity through 30 months; however, cEH activity increases during the first 9 months of life followed by a steady decrease through 30 months of age [166].

Glucuronyl Transferase

Glucuronidation is also a major pathway for the metabolic elimination of parent compounds (such as 4-ipomeanol) or primary metabolites (such as 1-naphthol) and as such can provide important means of protecting extrahepatic tissues from toxicants [167]. As with the other phase II enzymes discussed in this chapter, glucuronyl transferases are widely distributed throughout tissues, with the highest activity found in the liver. These enzymes have been described in the lungs of adult rabbits [168], dogs [165, 169], humans [164], and sheep [170]. Similar to CYP

monooxygenases, the distribution of UDP-glucuronyl transferase is restricted to the bronchial epithelial cells, Clara cells, and type II pneumocytes in the rat lung. There are reported species-specific differences in activity levels. UDP-glucuronyltransferase activity has been shown to be evenly distributed throughout the respiratory tract of the dog and is similar to activity levels found in the liver [165]. In the human [171], pulmonary UDP-glucuronyl transferase activity is considerably lower than hepatic activity. In contrast to the detailed studies of the development of hepatic glucuronyl transferases, there is only one report on the developmental expression of this enzyme in the lung. In goats, pulmonary UDP-glucuronyl transferase activity has been reported to be lowest at birth and highest in adults. However, the pattern of enzyme expression also varies by substrate used [127].

Miscellaneous Enzymes

In addition to the four enzyme systems already mentioned, there is a small amount of information available concerning the expression of two other enzymes during lung development: flavin-containing monooxygenases and sulfotransferases [139, 172]. Flavin-containing monooxygenases are important oxidative metabolizing enzymes and have much in common with the cytochrome P450 monooxygenases. They have similar molecular weights, are localized in SER, have the highest expression in liver, require NADPH, and have multiple isozymes [173, 174]. To date, flavin-containing monooxygenases have only been studied during lung development in rabbits [139]. Activity, protein, and mRNA are all expressed as early as 25-day gestation (canalicular stage). Flavin-containing monooxygenase expression is high prenatally (except for an unexplained decrease at 28-day gestation), drops immediately after birth, and then steadily increases throughout postnatal lung development. This expression pattern matches that of CYP2B4 and CYP4B1 in the rabbit lung [139]. Sulfation is a major detoxification pathway, resulting in a highly water-soluble product [175, 176]. There are two major subfamilies: phenol sulfotransferase and hydroxysteroid sulfotransferase, each of which has different substrate specificities [177]. Human pulmonary hydroxysteroid sulfotransferase, which sulfates steroids and cholesterol, is found in low levels early in gestation (around 56-day gestation) and expression peaks at 1 year after birth [172]. Hydroxysteroid sulfotransferase is expressed in most ciliated, nonciliated, and basal airway cells, but not in mucus-secreting cells. Human phenol sulfotransferase is highly expressed and widely distributed in fetal lung [172, 178]. After birth, expression decreases and the distribution is restricted to the proximal airways.

Cellular Injury in Response to Toxicant Exposure

The respiratory system is a target for a wide range of toxic environmental contaminants. While the acute and chronic effects of a large number of these substances have been well characterized for the respiratory system of young adult mammals,

there is significantly less known about the impact of these lung-targeted compounds on the developing and aged respiratory system. Recent studies have begun to identify mitochondria a target of many of these compounds and also highlight their role in the progression of cellular injury. This section summarizes what is currently known about three classes of these compounds, addressing first environmental tobacco smoke, a well-recognized lung toxicant mixture, bioactivated environmental contaminants, and oxidant gases, all of which have been shown to affect the developing and/or aged lung.

Environmental Tobacco Smoke

The health consequences of exposure to environmental tobacco smoke (ETS) among children are the ongoing subject of much public health concern. Animal studies are very valuable because the indirect effects of in utero ETS exposure can be separated from the direct effects of postnatal ETS exposure.

Exclusively in utero exposure to ETS has been shown to accelerate the developmental pattern of Clara cell secretory protein expression in the rat [179], suggesting a potential acceleration of airway epithelial differentiation in the lung. Whether this accelerated development is maintained after birth is unknown. In utero exposure does not increase cytochrome P450 gene expression unless it is combined with an early postnatal exposure [180].

Exclusively postnatal ETS exposure in rats did not alter Clara cell secretory protein expression [131]. Postnatal ETS exposure did, however, decrease cell kinetic activity in distal airways and increase cytochrome P450 1A1 protein distribution throughout the airway tree in postnatal ETS chronically exposed rats [131]. These changes were maintained for up to 100 days (with ongoing ETS exposure). Separate from the other compounds in ETS, nicotine exposure during gestation and lactation has been shown to increase CYP2A3 and CYP2B1 mRNA [181]. Acute postnatal exposure to ETS in juvenile ferrets has been shown to increase the ability of the lungs to metabolize (−)-trans-benzo[a]pyrene-7,8-dihydrodiol [182]. When slightly older rats (weanling age) are exposed to tobacco smoke, emphysematous changes are reported in their lungs [183]. Postnatal exposure to ETS has also been reported to affect the neurophysiologic responses of the lung. Chronic ETS exposure during the period of postnatal lung development in guinea pigs has been shown to increase lung C-fiber sensitivity [184]. In addition, ETS can increase the sensitivity of C-fiber-activated neurons in the nucleus tractus solitarii (NTS) of the central nervous system [185, 186].

A combination of in utero and postnatal exposure to ETS appears to have the greatest effect on developing lungs. Rats exposed to both in utero and postnatal ETS have decreased lung compliance, increased reactivity to methacholine, and an increase in the number of neuroendocrine cells per cm of basal lamina [187]. These changes are not seen in rats exposed to ETS in utero only or postnatal only. The increased airway hyperresponsiveness that is set up during postnatal exposure does not resolve even after an extensive period of no exposure to ETS [188].

In nonhuman primates, in utero plus postnatal exposure to ETS increases pulmonary adenyl cyclase activity [189]. Recent studies have focused on the effects that in utero exposure to ETS may have on the etiology of asthma. Early life exposures to ETS in the nonhuman primate enhance local Th2 immunity by impairing normal Th1 immune maturation [190]. In the mouse, in utero ETS exposure results in altered gene expression in adult animals [191] and exacerbating adult responses to allergen challenges [192]. In humans, in utero ETS exposure has been correlated to increased wheezing and increased doctor-diagnosed asthma by 2 years of age [193]. Whether in utero-induced alterations are the result of direct or indirect effects of ETS is unknown at this time.

ETS exposure is associated with increased GSH levels in the ELF; however, as mice age, response capacity is greatly reduced [96]. Unlike the developing lung, studies evaluating the effects of ETS on the adult and aged lung are limited; however, a large body of literature has found associations between cigarette smoking, premature aging, cancer, and COPD. Aged rats have a reduced ability to resist cigarette smoke-induced oxidative damage while still maintaining ability to activate PAHs to their carcinogenic form, shifting the balance to favor carcinogenesis with advanced age [90]. Senescent mice and senescence-accelerated mouse models have both been shown to have enhanced susceptibility to cigarette smoke [194–196]. Aging in the lung is believed to be accelerated by reactions between of components of cigarette smoke and extracellular matrix proteins to form covalent adducts that result in advanced glycation end products [116]. Advanced glycation end products (AGEs) play a role in aging and degenerative diseases due to their ability to cross-link proteins, especially structural proteins such as collagen [197]. Smoking has been shown to accelerate maturation of the fetal lung, impair lung growth, and accelerate age-related declines in FVC and FEV1 [198]. Reductions in FEV1 were also shown for cigar and pipe smokers who inhaled; however, non-inhaling pipe smokers had declines similar to nonsmokers [199].

Cigarette smoke constituents, such as phenols, aldehydes, and aromatic compounds, have been shown to accumulate in the mitochondria and disrupt ETC function and ATP production [200]. In a murine model for asthma, ETS exposure increases levels of ROS and lipid peroxides while decreasing mitochondrial enzymes [201]. In cell culture, cigarette smoke extracts (CSE) have been found to decrease membrane potential and ATP levels [202, 203]. In A549 cells, CSE increases intracellular ROS production; however, in cells the functional mitochondria (A549-rho0) have been depleted, suggesting that CSE increases mitochondrial ROS production. CSE also increases caspase-dependent apoptosis [202].

Bioactivated Compounds

The lungs of mammals are selectively injured by a host of chemically diverse agents including aromatic hydrocarbons, furans, halogenated ethylenes, and indoles [136]. Many of these agents target airway epithelium, especially Clara cells. In all cases, the metabolic activation of the chemically inert parent

compounds has been demonstrated to be an important factor in selective lung injury. It is generally assumed that the Clara cell is most susceptible by virtue of the high expression of cytochrome P450 monooxygenases in this cell type. Despite extensive documentation of Clara cell susceptibility to P450-mediated cytotoxicants in the lungs of adults [106, 120, 121, 202, 204–211], little is known about the susceptibility of undifferentiated and developing cells in the neonate to these compounds. The studies that are available regarding neonates counterintuitively suggest that lower pulmonary P450 activity is associated with greater susceptibility to P450-activated toxicants [128, 138, 212–214]. In utero exposure to bioactivated compounds produces embryotoxic or teratogenic effects, including chromosomal aberrations [215–220]. The latter appears to be the case for a number of pro-carcinogens which when given to pregnant mothers produce Clara cell tumors in adult offspring [219, 221, 222].

The herbicide dichlobenil specifically injures olfactory nasal mucosa in fetal and neonatal mice just as it does in adult mice [223]. The toxicity of dichlobenil increases in neonatal mice with the development of Bowman's glands. In fetal mice, there is more irreversible binding of ^{14}C-dichlobenil in the nasal cavity when dichlobenil is given to the dam as opposed to injected directly into the fetus. This suggests that maternal metabolism of dichlobenil may be important in the fetus.

While the toxicity of dichlobenil increases with development of the target organ, this is not always true for the other bioactivated compounds in the developing lung. Neonatal rabbits are much more sensitive to the P450-bioactivated furan 4-ipomeanol as compared to adult rabbits [213]. Distal airway epithelium of neonatal rabbits is injured at doses that do not appear to affect the adult rabbit airway epithelium. This would seem to be a contradiction because development of the P450 system is a postnatal event, and in neonatal rabbits, P450 activity is very low. This phenomenon is not restricted to the rabbit. Studies have shown that neonatal mice are also more susceptible than adult mice to the Clara cell cytotoxicant naphthalene [212] and neonates of both rats and mice are more susceptible to 1-nitronaphthalene than are adult animals [138]. Both 4-ipomeanol [224] and naphthalene [225, 226] have been shown to alter mitochondrial morphology in nonciliated cells in the terminal bronchioles of mice. In the hours and days after injury caused by either 4-ipomeanol or naphthalene administration, increase in the mitochondrial swelling has been noticed, suggesting that mitochondria may be a target of naphthalene metabolites, although studies to directly investigate this have not been performed.

In vitro metabolism studies of neonatal and adult airways show that P450 activity is lower in neonatal mouse lung than it is in adult mouse lung [129]. Gender may also play a role in heightened postnatal sensitivity to pulmonary toxicants. Weanling male and female mice are reported to be more susceptible to 1,1-dichloroethylene-induced pulmonary injury than adult male mice, but not more susceptible than adult female mice [227]. The exact mechanisms of these increases in sensitivity of postnatal animals have yet to be clearly defined. In some cases, levels of injury positively correlate with specific P450 monooxygenase activity [227], while in other cases phase II enzyme activity may be key to increased susceptibility [148]. The mechanisms may also involve as yet undefined factors specific to differentiating cells such as the important role of mitochondria in cellular injury.

Oxidant Gases

Lung injury occurs when concentrations of inhaled oxidant gases exceeds the detoxification capacity of the lung. The pulmonary effects of oxidant gases are dependent on a number of factors, including the age at exposure; chemical properties of the gas, which include solubility and reactivity; and the effective dose which is in part dependent upon ventilatory rates, concentration, and duration of exposure [228–230].

In contrast to our understanding of P450-activated lung toxicants, the susceptibility of the lungs of postnatal animals to oxidant gases is much better understood. For the best-studied oxidant gas environments (hyperbaric oxygen, ozone and nitrogen dioxide), two fundamental characteristics have been defined. First, in general, postnatal animals, prior to weaning, are less susceptible to pulmonary injury than are adults. Second, exposure to oxidant gases retards postnatal maturation of the lung.

The tolerance of postnatal animals to hyperoxia appears to be species-specific [70, 231, 232] and is based on differences in (1) the ability of neonatal animals to elevate pulmonary antioxidant defense systems in response to hyperoxic stress [233–235], (2) the composition of lung polyunsaturated fatty acids [236], or (3) the presence of antioxidant compounds, including iron chelators [82]. A common factor appears to be the ability to increase the intracellular GSH pool and to upregulate the enzymes whose antioxidant functions depend on it, including SOD, catalase, GPx, and glucose 6-phosphate dehydrogenase [70, 232, 237–240]. Undernutrition and premature weaning have also been shown to alter susceptibility [82, 241, 242]. Pharmacologic intervention by administration of steroids (dexamethasone) or endotoxin attenuates neonatal susceptibility to hyperoxia but has a mixed effect on antioxidant enzyme activity (endotoxin elevates them, dexamethasone does not) [243, 244]. Hyperoxia has been shown to delay lung morphogenesis, including alveolarization and vascularization [245–248], and differentiation of Clara cells in postnatal rats [249]. Studies in mice have shown that the decreased alveolarization is due to arrested alveolar development that can be attributed to decreased respiratory control ratios and decreased O_2 consumption, suggesting impaired mitochondrial function [250]. Treatment with retinoic acid does not prevent hyperoxia-induced alterations in alveolarization; however, it does result in later improvement in alveolarization [251, 252]. Excessive collagen deposition (fibrosis) that is associated with the decreased alveolar and capillary development is preceded by an increase in connective tissue growth factor (CTGF) in neonatal rats [253]. Despite alterations in lung development, neonatal rats have been reported to survive hyperoxia longer than adult rats. This may partially be due to the fact that in neonatal rats, there is a delay in pulmonary neutrophil influx [254]. Compared to adult rats, neonatal rats have fewer overall lung tissue neutrophils, even though they have higher levels of neutrophils in bronchoalveolar lavage. This suggests that neonatal rats retain fewer neutrophils than adult rats [254]. When neonatal rats exposed to hyperoxia were treated with antibodies to cytokine-induced neutrophil chemoattractant-1 (CINC-1) to block neutrophil influx, they had increased lung compliance and no change in

alveolar volume or surface density as compared to control antibody-treated neonates [255]. In addition, blocking neutrophil influx reduces DNA damage in the neonatal lung [256]. The retardation of alveolar development in neonatal lung may also be related to the timing of the hyperoxia and subsequent exposure to leukotrienes [257] or a reported increase in the number of apoptotic cells in the lungs of hyperoxia-exposed neonates [258].

Rats exposed to hyperoxia during the first 7 days of life have shown increased volume density of mitochondria in both ciliated and Clara cells as well as reduced nuclear density of bronchiolar epithelial cells immediately after exposure [259]. However, recovery in filtered air resulted in a reversal of these changes. In adult rats exposed to 70 % oxygen, mitochondria have shown increased ROS in the matrix, suggesting that an increase in the oxidative burden is mitochondrially derived [260]. After exposure, an increased number of cells entered a premature senescent state, which may be due to the increased ROS production resulting in mtDNA damage and mitochondrial dysfunction.

For some parameters, neonates appear to be less susceptible to inhaled oxidant gases due to fewer alterations in pulmonary enzymes and markedly decreased cellular injury in the central acinus as compared to adults [261–265]. Weaning appears to be the critical time point for changes in responsiveness. Pre-weanling animals are much less sensitive than post-weaning animals [262, 264, 265]. As in hyperoxia, ozone exposure reduces the postnatal morphogenesis of the gas-exchange area [266], impairs bronchiolar formation [267], and retards the differentiation of the mucociliary apparatus of proximal airways [268]. Nonhuman primates exposed to cyclic episodes of ozone during the first 6 months of life were found to have four fewer non-alveolarized airway generations, hyperplastic bronchiolar epithelium, and altered smooth muscle bundle orientation in terminal and respiratory bronchioles compared to filtered air-exposed control animals [269] and hyperinnervation of the pulmonary epithelium [270]. The molecular mechanism behind the abnormal development of distal conducting airways in animals exposed to ozone may be related to the depletion of perlecan in the basement membrane zone [271]. Perlecan is a proteoglycan responsible for many functions, in particular, regulation of growth factor trafficking between cells of the epithelial-mesenchymal unit [272, 273]. Ozone-induced depletion of perlecan from the basement membrane zone in trachea has been shown to be associated with altered regulation of FGF-2 signaling [271, 274]. Depletion of perlecan would also effect regulation of the other growth factors that bind to perlecan which also include FGF-1, FGF-7, PDGF, hepatocyte growth factor, and heparin-binding EGF, VEGF, and TGF-β [275]. The functional consequences of deregulation of these collective molecules are significant since they are the basis for much of the cell-cell interactions in the epithelial-mesenchymal trophic unit responsible for development of the airway. The dysregulation of the epithelial-mesenchymal trophic unit may play a role in explaining the epidemiological findings regarding children who are exposed to long-term outdoor air pollution. In Southern California, an association has been found between long-term exposure of children to outdoor air pollution and deficits in lung function as these children become adolescents and young adults [276–279]. Children growing up in Mexico City, which has very high

levels of ozone and other air pollutants, have respiratory abnormalities such as hyperinflation and increased interstitial markings of the lung [280–282].

A potential mechanism for the age-related differences in ozone-induced injury may be the way in which neonates control their ventilation during exposure. Neonatal rats have higher baseline minute ventilation than adult rats. During ozone exposure, adult rats will reduce their minute ventilation even further, while neonatal rats will not [283]. This indicates that neonatal rats receive a higher delivered dose of ozone than adults and may explain increased indices of acute injury such as increased bronchoalveolar lavage protein, neutrophils, and prostaglandin E_2 levels [284, 285]. What can be concluded from these studies with oxidant gases is that two aspects of postnatal lung development, which involve Clara cells, the rate of differentiation of bronchiolar epithelium, and the organization and differentiation of the centriacinus, are impeded by oxidant gas injury. Whether this is true for other classes of pulmonary toxicants such as organic chemicals metabolized by the cytochrome P450 system has not been investigated.

Clinical and epidemiological studies have clearly shown an association between acute ozone exposures and increased respiratory-related hospital visits in elderly patients [286, 287]. In controlled acute ozone exposure studies in human subjects, younger healthy adults showed greater adverse effects than older individuals [288]. However, after long-term exposures, the effects of ozone on the human lung appear to be greater with increasing age [289]. In animal models, ozone exposure has resulted in greater initial injury in aged rats as indicated by increased IL-6 in the lavage fluid and increased ^{18}O-ozone incorporation in tissues [104]. The cellular and biochemical effects of ozone are also greater in senescent rats as compared to juvenile and adult rats [104, 290]. Ozone has been shown to enhance lung mitochondria ROS production [40]. Measures of mitochondrial function, including O_2 consumption, RCR, and H_2O_2/O_2 ratios, have not been altered by ozone in young or adult rats; however, aged rats have shown increased state 4 respiration, decreased RCR, and increased H_2O_2 release as compared to age-matched controls [40]. Ozone has been shown to increase antioxidant enzyme activity, especially SOD and GPx, in the lung; however, these increases do not appear to provide sufficient protection to prevent disruptions to mitochondrial function or accumulation of 8-oxodG (an index of DNA damage) lesions in the tissue [40].

Conclusions

The impact of lung-targeted toxicants on the respiratory system of developing and aging animals is not well defined, let alone the role that mitochondria may play in the cascade of factors involved in cellular injury. The pattern of lung development itself may play a significant role in modulating the toxic response since significant portions of lung morphogenesis and cytodifferentiation occur during the postnatal period. The enzyme systems responsible for bioactivation and detoxification differentiate during the perinatal period, with the majority of differentiation activity

occurring for an extended period of time after birth. In addition, each enzyme system has different pattern of differentiation during pre- and postnatal lung development. A pulmonary toxicant may have its impact by altering the processes of morphogenesis and cytodifferentiation resulting in differential expression or organization of the lung in the adult, a change that could be due to alterations in mitochondrial function. Factors such as the stage of morphogenesis and differentiation of various sub-compartments of the lungs during the time of exposure may also significantly increase the severity of the toxic response. While there is extensive literature on the toxic potential of a wide range of environmental contaminants when the exposure is directed toward young, healthy adults, there is a lack of complete information regarding the toxic response of the respiratory system during development or during aging. The majority of studies suggest that (1) the respiratory system in pre- and postnatal animals is more susceptible to injury from lung-directed toxicants than it is in adults of the same species and (2) the differences in toxic response to respiratory-targeted compounds among species are amplified when responses are evaluated during lung development. Current data suggest that the human population most at risk to respiratory-targeted environmental contaminants endanger fetuses, neonates, and the elderly and that their risk is significantly higher than the risks faced by the adult population. A review of the literature indicates that there is evidence of mitochondrial involvement in the cellular injury caused by the environmental toxicants during both lung development and aging. Whether the mitochondrial alterations are a critical early step in the injury or an end result of a cascade of processes is not clear at this time.

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