Published in final edited form as:

Free Radic Biol Med. 2007 April 1; 42(7): 897–908.

Hyperoxia-induced signal transduction pathways in pulmonary epithelial cells

Tahereh E. Zaher^{a,b}, Edmund J. Miller^c, Dympna M. P. Morrow^b, Mohammad Javdan^b, and Lin L. Mantell^{a,b,*}

a Department of Pharmaceutical Sciences, St. John's University College of Pharmacy, Queens, NY 11439

b Cardiopulmonary Research, The Feinstein Institute for Medical Research, North Shore-LIJ Health System, Manhasset, NY 11030

c Surgical Immunology, The Feinstein Institute for Medical Research, North Shore-LIJ Health System, Manhasset, NY 11030

Abstract

Mechanical ventilation with hyperoxia is necessary to treat critically ill patients. However, prolonged exposure to hyperoxia leads to the generation of excessive reactive oxygen species (ROS), which can cause acute inflammatory lung injury. One of the major effects of hyperoxia is the injury and death of pulmonary epithelium, which is accompanied by increased levels of pulmonary proinflammatory cytokines and excessive leukocyte infiltration. A thorough understanding of the signaling pathways leading to pulmonary epithelial cell injury/death may provide some insights into the pathogenesis of hyperoxia-induced acute inflammatory lung injury. This review focuses on epithelial responses to hyperoxia and some of the major factors regulating pathways to epithelial cell injury/death, and proinflammatory responses upon exposure to hyperoxia. We discuss in detail some of the most interesting players, such as, NF- κ B, that can modulate both proinflammatory responses and cell injury/death of lung epithelial cells. A better appreciation for the functions of these factors will no doubt help us to delineate the pathways to hyperoxic cell death and proinflammatory responses.

Keywords

Hyperoxia; ROS; Epithelial cells; Lung injury; Signal transduction; NF- κ B; MAPK

Introduction

Oxygen is required to sustain aerobic life. Supraphysiological concentrations of O₂ (hyperoxia) are routinely used to prevent or treat hypoxemia and acute respiratory failure [1,2]. However, prolonged exposure to hyperoxia can result in tissue damage in many organs, including lungs, and lead to the development of both acute and chronic lung injury [3,4]. The lung is particularly vulnerable to exogenous oxidative inhalants because of its large epithelial surface and anatomic location. Hyperoxia-induced damage is characterized by an extensive inflammatory response,

*Correspondence author: Lin L. Mantell, Department of Pharmaceutical Sciences, St. John's University College of Pharmacy, 108/SB28 St. Albert Hall, 8000 Utopia Parkway, Queens, New York 11439, Tel: 718-990-5933, Fax: 718-990-1877, Email: mantelll@stjohns.edu

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destruction of the alveolar-capillary barrier, impaired gas exchange and pulmonary edema [5].

Significant progress has been made in delineating the regulatory and functional elements of hyperoxic lung injury. Pulmonary epithelial cells are essential in maintaining the integrity of the alveolar-capillary barrier and serve as a first line of defense against a variety of insults, including excessive oxygen. In hyperoxic animals, however, injury and death of pulmonary epithelium is prominent [6].

The molecular mechanisms leading to pulmonary cell death in hyperoxia are regulated at several levels. First, oxygen toxicity is believed to be mediated by the production and accumulation of excessive ROS, such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO\cdot$) at levels exceeding the capacity of the lung antioxidant defense mechanisms [7–9]. The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system, in conjunction with mitochondria, are the major sites of ROS generation during hyperoxia [10–13].

Excessive ROS can directly damage cellular macro-molecules, resulting in cell cycle arrest and/or cell death [14–16]. In addition, exposure to hyperoxia triggers an inflammatory response, which exacerbates oxidative toxicity [17]. Upon exposure to hyperoxia, ROS evoke pulmonary cells to increase the secretion of chemoattractants and other proinflammatory cytokines that leads to leukocyte recruitment to the lung. Recruited leukocytes, including neutrophils and monocytes, accumulate within the pulmonary circulation, interstitium and air spaces and are significant sources of additional ROS, as well as proteinases, and antimicrobial peptides [18–21]. Therefore, the interactions between ROS and leukocytes establish a vicious cycle that initiates and/or exacerbates lung injury. In this series of reviews, Dr. Downey and colleagues discuss the role of ROS in neutrophil functions and the effects of inflammation on lung injury. In this review, we focus on the recent advances on signaling pathways that modulate epithelial responses to hyperoxia and their implications in inflammatory responses in hyperoxic lungs.

Pulmonary Epithelial Cell death in Hyperoxia

Pulmonary epithelial cells are essential in maintaining the homeostasis of pulmonary functions. Damage to these cells can lead to dysfunctions and injury of the lung [22,23]. Under physiological conditions, proliferation and death of lung epithelial cells are under stringent control. However, the production of excessive ROS during exposure to prolonged hyperoxia can trigger the injury and death of epithelial cells in hyperoxic animal lungs and in cell culture model systems [10,11,24–27].

The morphological changes in pulmonary cells in hyperoxic lungs have been extensively studied [28]. In a variety of animal models, the earliest signs of hyperoxic cell injury are found in capillary endothelial cells, which exhibit characteristics of necrosis, such as swelling of the cytoplasm and intracellular organelles, and rupture of cytoplasmic and nuclear membranes [29–31]. The pulmonary epithelium is known to have a superior antioxidant system and is relatively resistant to oxidative stress. Nonetheless, similar morphometric changes as those occurred in endothelial cells are observed in some of the pulmonary epithelial cells in lungs of animals exposed to hyperoxia. Necrotic morphologies are clearly part of the characteristics of lung epithelium in hyperoxic lungs [28,32,33].

In the mid-1990s, with the increased understanding of apoptosis and the available molecular approaches, we and others addressed the question of whether lung epithelium undergoes programmed cell death in hyperoxic lungs [32,34]. These studies reveal that apoptosis of

epithelial cells occurs in hyperoxic lungs. The presence of apoptosis of hyperoxic epithelium was evaluated by the presence of oligonucleosomal DNA fragments as assessed by terminal dUTP nick end labeling (TUNEL) analysis, and specific internucleosomal DNA laddering, immunohistochemical and biochemical assays for apoptotic gene expression and activities [12,25,26,35–39]. In addition, increased caspase activities have also been observed in hyperoxic lungs by several investigators [12,38,40]. Caspase activation is considered to represent the onset of irreversible apoptotic pathway. Caspase-1, an important mediator in both apoptosis and inflammation, is activated in fractionated lysates of hyperoxic mouse lungs [34]. Exposure of caspase-3 deficient mice to hyperoxia for 72 hours induced less apoptotic cell death of lung epithelial cells, compared to that of the wildtype mice [12]. This suggests that caspase-3 activation is one of the critical steps in hyperoxia induced cell death. In addition, inhibition of caspase-8, achieved by over expression of Flip, conferred substantial resistance to oxygen toxicity [40]. Furthermore, type II alveolar epithelial cells isolated from hyperoxia-exposed rat lungs exhibited evident apoptotic characteristics [37]. These studies support the notion that apoptosis of lung epithelium is a prominent feature of hyperoxic lung injury. Together with the observations on necrosis in the early studies, these studies indicate that multimodal cell death occurs in lung epithelial cells upon exposure to prolonged hyperoxia.

Interestingly, some bronchial and alveolar epithelia in hyperoxic lungs display characteristics of both necrosis and apoptosis [40,41]. For example, it was observed that the plasma membranes of some epithelial cells with condensed chromatin were disrupted. In addition, activation of apoptotic genes, such as Bid and caspase-8, occurs in epithelial cells undergoing hyperoxia-induced necrosis [25,40]. These studies lead to the following hypotheses: 1) exposure to hyperoxia leads to cell death of lung epithelial cells through a two stage process: (i) excessive ROS initiate the onset of apoptotic pathways (ii) the resulting dysfunctional mitochondria prevent the completion of apoptosis, leading to necrotic cell death; 2) alternatively, lung epithelium in prolonged hyperoxia undergoes cell death in a unique manner that is different from either apoptosis or necrosis. Regardless how epithelial cells die in hyperoxia, it is apparent that identification and inhibition of pathways that mediate hyperoxic epithelial cell death would help us to reduce epithelial dysfunction occurring during hyperoxic lung injury.

The lung is complex, comprised of more than 40 different types of cells. With the current approaches available for animal model systems, it is challenging to delineate signaling pathways leading to cell death occurring specifically in pulmonary epithelium. Therefore, we and others have addressed this issue using *in vitro* model systems of cultured lung epithelial cells [42,43]. Oxidative stress generally induces apoptosis in a relatively short period of time (hours) [32]. However, hyperoxia predominately induces nonapoptotic cell death of cultured epithelial cells over relatively long periods of time (days) even when the concentration of oxygen is 95% [32,33,44]. Transformed human and mouse lung epithelia exhibit swollen mitochondria, and enlarged nuclei with no apparent chromatin condensation following the exposure to hyperoxia [32]. Together with the specific pathways activated in these cells, this type of cell death was referred to as oncosis (rather than necrosis), since specific pathways are not typically associated with necrosis [40,43]. Similar morphological changes were also observed in normal human bronchial epithelial (NHBE) cells in response to hyperoxia [45], suggesting that hyperoxic oncosis is not unique to transformed lung epithelial cells. In addition to the swollen characteristics, neither elevated caspase-3 activity, nor phosphatidylserine translocation, were detected in lung epithelium, exposed to 95% O₂, although caspase-8/Bid pathway can modulate this type of cell death in both animals and cultured human alveolar type II cell-like (A549) cells [40,46]. In contrast to these observations, studies by Zhang *et al.* show that murine lung epithelial (MLE-12) cells undergo apoptosis with increased activities of caspase 9 and 3 as detected by the CasPACE assay system [12]. Further support for the involvement of caspases is derived from the observation that, treatment of cells with specific

inhibitors of caspase 3 and 9, Z-DQMD-FMK and AC-LEHD-CMK respectively, caused significant inhibition of hyperoxic-induced apoptotic cell death [12]. A recent study further indicates that the early effects of hyperoxia (24–48h) on type II alveolar epithelial cells are characterized by caspase-mediated apoptosis, while the residual adherent cells exhibit non-apoptotic characteristics [47]. The discrepancies between these observations may be reflective of technical differences, such as different culturing conditions and diversities in these cell lines. Alternatively, they may be indicative of the complex nature of the pathways involved in hyperoxic cell death, which can be altered by many factors during lung injury. Most importantly, hyperoxic cell death of lung epithelium with either apoptotic or oncotic morphologies are pathway driven, and with some common for both modes of cell death. For example, pro-apoptotic factors Bid and Bax are up-regulated and cytochrome c is released into the cytosol in necrotic A549 cells [40]. The signaling pathways involved in hyperoxic epithelial cell death will be discussed in detail in the next section.

Expression of Regulatory Genes in Hyperoxic Cell Death

With the hope that one can mitigate injury by specifically blocking deleterious pathways that lead to hyperoxic epithelial cell death, a significant effort has been made in the identification of these biochemical pathways and cell death mediators. It is becoming clear that hyperoxia-induced epithelial cell death can be regulated by a variety of factors. The major subfamily members of mitogen activated protein kinases (MAPKs) include extracellular signal regulated kinase (ERK1/2), c-Jun N-terminal protein kinase (JNK1/2), and p38 kinase [48]. JNK1/2 and p38 have been shown to be inducers of cell death and/or inflammation following exposure of cells to different stimuli. The MAPKs also play a critical role in regulating hyperoxia-induced epithelial cell death as the activation of two members of MAPK, JNK and p38 is associated with hyperoxia-induced epithelial damage [12,46,49]. Using pharmacological inhibitors and expression of dominant negative constructs, we demonstrated that JNK and p38 mediate hyperoxic oncotic cell death [46]. In addition, apoptosis of MLE-12 cells, induced by oxidants was also mediated by JNK and p38 [46]. This suggests that although the downstream events of cell death are divergent, both hyperoxic-induced oncosis and oxidative apoptosis of lung epithelium share upstream pathways and activation of JNK and p38 MAPK is involved in oxidative death promoting pathways [46]. The regulatory role of JNK in hyperoxia-induced non-apoptotic death of MLE-12 cells was also observed by Li et al. [49]. In contrast to the *in vitro* studies, Morse and coworkers showed that both *Jnk1*^{-/-} and *Jnk2*^{-/-} mice exhibited more TUNEL positive signals than wildtype mice upon exposure to prolonged hyperoxia [50]. However, an increased level of phosphorylated JNK was observed in mouse lungs exposed to prolonged hyperoxia [151]. The discrepancy between these studies may be due to the essential roles of JNK in other aspects in response to hyperoxia or some intrinsic properties of these knock out animals. Future studies utilizing transgenic animals with an induced deficiency of JNK expression specifically in lung epithelial cells, would be important in determining the role of the JNK signaling pathway in hyperoxic epithelial cell death.

ERK1/2 is generally considered a promoter of cell proliferation. Thus, one could predict that ERK1/2 protects cells against hyperoxia-induced cell death. Indeed, a selective ERK1/2 inhibitor, PD-98059, abolished the protective effect of laminin against hyperoxic cell death of lung epithelium. Thus, Buckly *et al.* concluded that the ERK-dependent pathway mediates its effects by protecting the basement membrane against hyperoxic DNA strand breakage and death of type II alveolar epithelial cells [52]. A recent study also demonstrates that activation of ERK1/2 induced by either exposure to hyperoxia or over-expression of mitochondrial aldehyde dehydrogenase (mtALDH) attenuates hyperoxia-induced non-apoptotic death of lung epithelial cells through compensatory AKT cell survival signaling pathways [53]. Inosine, a naturally occurring purine with anti-inflammatory properties, was shown to reduce hyperoxic-induced cell death of alveolar epithelium by increasing ERK1/2 activation [54]. However,

Zhang et al reported that ERK1/2 activation potentiated hyperoxia-induced apoptosis of MLE-12 cells and treatment of mice with PD98059, a pharmacologic inhibitor of ERK1/2, effectively attenuated TUNEL and caspase 3 staining in mouse lungs [12]. Similar results were observed in cultured macrophages exposed to hyperoxia [51]. Thus, the role of ERK1/2 in hyperoxia, either as a promoter or inhibitor, may be cell type- and culturing condition-dependent. Taken together, these studies indicate that exposure to hyperoxia induces activation of multiple MAPK pathways, which play critical roles in modulating and resolving hyperoxia-induced epithelial cell death [55].

Currently, the upstream and downstream effectors of MAPK leading to hyperoxic cell death of lung epithelium remain to be fully elucidated. Some of the potential candidates include Fas, p53, caspases, Bcl-2 family members, cytokines (IL-6, IL-11, IL-1 β and TNF- α), growth factors (Vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF) and keratinocytes growth factor (KGF)), heme oxygenase 1 (HO-1), and Toll-like receptor 4 (TLR4) [56,57,63]. First, exposure to hyperoxia induces an altered expression of many of Bcl-2 family members, including both proapoptotic (Bax, Bad, Bak) and antiapoptotic (Bcl-k_L, Bcl-w, Bfl-1 and Bcl-2). These alterations may ultimately lead to an imbalance between pro- and anti-apoptotic Bcl-2 family proteins resulting in cell death [58,59]. In cultured macrophages, hyperoxia can suppress the pro-apoptotic BH3-only protein BimEL in an ERK-dependent manner [59]. In addition, over-expression of IL-6 and IL-11 can protect lung cells against hyperoxic cell death in both cell culture and animal models [60,61]. The mechanism for this protective effect is the maintenance of Bcl-2 levels through activating ERK pathway. Inhibition of mitogen activated protein/extracellular signal-related kinase (MEK-1), upstream of ERK1/2 pathway, can specifically attenuate the enhanced levels of Bcl-2 in hyperoxia, resulting in the abolishment of the cytoprotective effects by IL-6 and IL-11 against oxygen toxicity. Thus, studies in IL-6 over-expressing systems demonstrate that ERK is upstream of Bcl-2 in response to hyperoxia [62]. The mechanisms of HO-1-mediated cytoprotective effects against oxygen toxicity further support the role of MAPKs in mediating hyperoxia-induced signaling pathways. Carbon monoxide (CO), a byproduct of HO degradation of heme, protects epithelial cells from hyperoxic damage. Using dominant negative mutants and mice deficient in different MAPKs, Otterbein et al demonstrated the cytoprotective effects of CO is mediated by specific MKK3/p38 β pathways [57]. TLR4, a principal receptor for bacterial lipopolysaccharides (LPS), can be activated by noninfectious stimuli including hyperoxia [63]. TLR4^{-/-} mice exhibited an enhanced severity of lung injury and a higher mortality, suggesting a critical role for TLR4 in susceptibility to oxygen toxicity. Interestingly, the susceptibility of TLR4^{-/-} mice to hyperoxia correlates with the inability to up-regulate Bcl-2, Akt and HO-1 compared to the wildtype mice [65]. ERK activation may play essential roles in the maintenance of Bcl-2, Akt and HO-1 (the survival genes) as demonstrated and discussed [66,67].

The downstream effectors of MAPKs in hyperoxia also include other redox transcription factors, such as nuclear factor-kappa B (NF- κ B), AP-1 and NF-E2-related transcription factor 2 (Nrf2). The interaction between MAPKs and these transcription factors will be discussed in the following section. Clearly, the MAPK-mediated signaling pathways activated in hyperoxia need to be further delineated and characterized in animal models of hyperoxic lung injury. However, there is no doubt that these pathways are intimately involved in the signaling networks that are activated in hyperoxia and play critical roles in mediating cell death as well as cell survival upon exposure to prolonged hyperoxia.

Redox transcription factors in Pulmonary Hyperoxic cell death

Hyperoxic lung injury is associated with the activation of number of transcription factors, including NF- κ B, AP-1, and signal transducers and activators of transcription (STAT) [56]. Emerging evidence has demonstrated that these transcription factors play important roles in

regulating pulmonary responses, including pulmonary cell death following hyperoxic exposure [16,68]. Understanding the roles of these transcriptional factors in modulating the death of pulmonary epithelial cells may shed light on the pathogenesis of hyperoxia-induced acute lung injury.

NF- κ B, a multi-subunit transcription factor, regulates expression of genes involved in inflammation, infection, and stress in response to various stimuli, including inflammatory cytokines, chemotherapeutic agents, and oxidative stress [69–71]. In hyperoxic lungs, activation of NF- κ B is detected in multiple cell types, such as alveolar macrophages, epithelial lung cells, human pulmonary artery endothelial cells and the monocytic THP-1 cell line [16, 45,70,72–78]. In addition, hyperoxia can also potentiate LPS and IFN- γ induced NF- κ B activation [72].

Studies over the last ten years have demonstrated that NF- κ B mediates a survival mechanism for protecting cells against apoptotic cell death [79]. We previously showed that pre-exposure to either hyperoxia or TNF- α activates NF- κ B in lung epithelial cells and this activation confers epithelial cells more resistant to subsequent oxidant-induced cell death [70]. We further examined the protective effect of NF- κ B against hyperoxic-induced, non-apoptotic cell death of lung epithelium, by inhibiting NF- κ B activation through either a pharmacological approach or genetic manipulation. Lung epithelial A549 cells with deficient NF- κ B were exposed to prolonged 95% O₂, which induces oncosis [45,71]. These transgenic A549 cells stably over-express dominant negative inhibitor of NF- κ B (IkB) mutant DNA construct and had less than 50% NF- κ B activity compared to the control clones that over-express the DNA vector alone [45]. In comparison to control vector cells, dominant negative IkB mutant A549 cells exhibited significant higher sensitivity to hyperoxia-induced non-apoptotic cell death. This finding was confirmed in primary normal human bronchial epithelial cells (NHBE), as pretreatment with an irreversible NF- κ B inhibitor (BAY 11-7082) accelerated hyperoxic non-apoptotic cell death [45,80]. These data indicate that NF- κ B modulates susceptibility of lung epithelial cells to hyperoxia-induced nonapoptotic cell death. The role of NF- κ B activation in hyperoxic lung injury is elegantly highlighted by an in vivo study by Yang et al [151]. Using NF- κ B/luciferase transgenic mice to visualize NF- κ B activation, Yang et al observed that neonatal mice exhibited much more pronounced and sustained NF- κ B activation. Furthermore, they demonstrated the protective role of NF- κ B against hyperoxic epithelial injury in the neonatal mice using both pharmacological and genetic approaches to inhibit NF- κ B activation.

Degradation and resynthesis of IkB α , a major intracellular inhibitor of NF- κ B activation, may serve as a central feedback mechanism to attenuate ongoing NF- κ B activation during prolonged exposure to hyperoxia. Exposure to hyperoxia induces IkB α degradation in lung epithelial cells [45,71,81]. However, prolonged exposure to hyperoxia triggers a negative feedback mechanism. In NHBE cells, the extent of IkB α degradation was diminished and hyperoxia-induced NF- κ B activation was blunted after 72 hour's exposure to 95% O₂ [45]. This feedback mechanism might be a critical factor in explaining why lung epithelial cells eventually die in hyperoxia even though NF- κ B activation was initiated by hyperoxia [16,45]. Together with its role in sensitizing the airway epithelium to oxidative apoptosis, the regulation of the steady-state levels of IkB α might be an important modulator of oxidative lung injury and pulmonary remodeling [70,77,82–85].

The role of NF- κ B in protecting epithelial cells against hyperoxic cell death may be attributed to its function in directly inducing the expression of anti-apoptotic factors and other signaling pathways promoting cell survival, as well as decreasing and/or arresting the expression of the hyperoxia-induced proapoptotic factors [35,78,86,87]. Cytoprotective enzymes such as manganese superoxide dismutase (MnSOD) and glutathione peroxidase are regulated by NF- κ B [45,65,88], which could help to explain the protective effect of NF- κ B against hyperoxia-

induced oncotic cell death. Indeed, lower basal levels of MnSOD were detected in NF- κ B deficient A549 cells, achieved by stably expressing a dominant negative I κ B mutant, compared to that in A549 cells with normal NF- κ B activity. The increased sensitivity to oxygen toxicity in these NF- κ B deficient cells is likely attributed to low levels of MnSOD [45]. In addition, Choi *et al.* reported that pre-treatment with hyperoxia reduced myocardial necrosis and apoptosis induced by ischemia/reperfusion injury. The protective effect was most likely the result of increased NF- κ B activation, because NF- κ B activation correlated with the elevated levels of Bcl-2, and inhibition of NF- κ B attenuated Bcl-2 expression [89]. These data suggest that NF- κ B is upstream of Bcl-2. In addition, this study confirmed our previous observation that pre-exposing A549 and human bronchial (16HBE) cells to hyperoxia for 24h can protect these cells from H₂O₂ induced cell death via NF- κ B activation [70].

The downstream effects of NF- κ B in hyperoxia also involve up-regulation of IL-6. The levels of IL-6 and IL-11 are significantly correlated with the protection of animals from hyperoxic cell death and acute lung injury [90,91]. Transgenic mice that over-express either IL-6 or IL-11 exhibited remarkable tolerance and survival advantages following exposure to hyperoxia compared to the wildtype mice [90–92]. These studies further demonstrated that IL-6 and IL-11 are critical in protecting animals against acute lung injury via reducing hyperoxic cell death. It has been reported that the activation of NF- κ B by oxidative stress plays an essential role in the expression of IL-6, which has the NF- κ B binding motif in the promoter region [93]. In addition to affecting ROS-mediated cell death, the protective role of NF- κ B in preserving tissue integrity in hyperoxic lung injury may also be attributed to its protective role against cell death induced by reactive nitrogen species, which are also elevated under hyperoxia [71,94].

In addition to NF- κ B, AP-1 is another transcription factor which has been shown to be involved in oxidant signaling, oncogenic transformation, immune responses, cellular differentiation, and apoptosis [95,96]. A number of studies indicate that AP-1 may be involved in the pathogenesis of hyperoxic inflammatory lung injury. In rats, the level of AP-1 activity is increased in the lung upon exposure to hyperoxia [46,97]. In addition, hyperoxia-induced oncosis of MLE-12 cells is associated with a sustained activation of AP-1 [46]. Furthermore, in MLE-12 cells over-expressing a dominant negative DNA construct of AP-1 (which inhibits AP-1 activation), exhibited enhanced survival in prolonged hyperoxia [46,72].

STATs are a family of multiple related polypeptides that participate in signaling pathways that mediate a wide variety of cellular and organ responses to growth factors and cytokines [98]. Transgenic mice, with the STAT3 gene selectively deleted in respiratory epithelial cell, exhibited significantly greater epithelial cell injury, inflammatory responses and mortality compared to the wildtype mice [99,100]. Heme oxygenase-1 (HO-1) is a cytoprotective enzyme and is highly inducible following exposure to hyperoxia and over-expression of HO-1 in pulmonary epithelial cells has been shown to protect animals and cells against oxygen toxicity [101]. Lee *et al.* reported that STATs may exert their protective effects through the induction of HO-1, as STAT binding elements are critical for hyperoxia-induced HO-1 gene expression [102]. In addition, mice that over-express the STAT3, exhibited delayed capillary leakage and neutrophil infiltration into the alveolar region [99]. The protective effect exhibited in these transgenic mice is mediated through inhibition of hyperoxia-induced synthesis and release of matrix metalloproteinase (MMP)-9 and MMP12 by neutrophil and resident alveolar cells [99]. Therefore, activation of the STAT3 pathway plays an important role in the prevention of hyperoxia-induced lung epithelial injury and inflammation by decreasing potentially harmful MMP production and excessive neutrophil influx into the lung.

Using elegant genetic approaches, Cho *et al.* have characterized the important role of the NF-E2-related transcription factor2 (Nrf2) in modulating susceptibility to hyperoxic lung injury [103]. Nrf2 can activate antioxidant response element (ARE)-mediated gene expression and is

activated upon exposure to hyperoxia. Mice deficient in Nrf2 suffer from marked more epithelial injury and loss of pulmonary functions compared to the wildtype mice [104].

Cross talk between transcription factors and MAPKs has been demonstrated by several studies [85,105–107]. NF- κ B, AP-1 and Nrf2 are three potential MAPK targets in response to oxidative stress. As discussed above, the death of epithelial cells involves the activation of all three major subfamilies of the MAPKs and transcription factors, NF- κ B, AP-1 and Nrf2 [28,56]. Inhibition of the MEK-ERK pathway activated NF- κ B, whereas inhibition of the MEKK-JNK pathways reduced NF- κ B activation under oxidative stress [85]. Strassheim *et al.* reported that ROS were involved in activation of nuclear translocation of NF- κ B, which is mediated by the activation of several kinases, including IkappaB α kinase (IKK), p38, ERK1/2, and Akt [105]. Inhibition of MAPK pathways can impede AP-1 mediated gene expression [108]. In alveolar epithelial C10 cells, hyperoxia increases translocation of Nrf2 from the cytoplasm to the nucleus, an indicator for its activation [109]. However, inhibition of ERK1/2 reduced hyperoxia stimulated phosphorylation of Nrf2, a necessary step for Nrf2 activation [109].

Therefore, one may conclude that exposure to hyperoxia induces a network of signal transduction involving redox transcription factors and MAPKs, with some promoting cell death while others inhibiting it. The ultimate fate of epithelial cells in prolonged hyperoxia depends on the overall balance of these pathways. Thus, to better design strategies to reduce hyperoxic epithelial cell death, one needs to further delineate these pathways/networks on a temporal and spatial level in both cell cultures and animal models.

Proinflammatory responses in hyperoxic lung injury

Hyperoxia-induced lung injury is the product of direct toxicity of ROS and indirect effects of the properties and products of activated resident and recruited inflammatory cells. The accumulation of leukocytes in lung tissues of patients with severe inflammatory lung injury is thought to significantly contribute to the development of acute lung injury [110]. It is believed that hyperoxia-induced acute lung injury is initiated by ROS. However, the more severe injury is associated with extensive infiltration of inflammatory leukocytes into the lung. In the lungs of animals exposed to prolonged hyperoxia, accompanied with pulmonary cell injury, proinflammatory responses are extensively pronounced [56]. For example, in the lungs of rats, exposure to 95% O₂ for 48 hours induced substantial neutrophil infiltration which coincides with the massive damage to endothelial and epithelial cell [111]. Besides the numbers, neutrophil properties, such as the half life and kinetics, could also be altered in a manner that ensures their prolonged, and uncontrolled presence in the lung during acute lung injury [112].

Similar to other tissues, influx of leukocytes into the lung is mediated by the degree of chemotactic activity, which is tightly controlled under physiological conditions. In hyperoxic lungs, however, chemotactic activity and leukocyte infiltration is highly elevated. In rats exposed to prolonged hyperoxia, there is a close temporal relationship between the high levels of pulmonary chemotactic activity and polymorphonuclears (PMNs) influx, the degree of lung injury and the mortality rate [18,113,114]. Proinflammatory cytokines are instrumental in determining the levels of chemotactic activity. In this series of reviews, Drs. Bahandri and Elias thoroughly discuss the increased expression of different cytokines in hyperoxic lungs. Some of these cytokines, such as interleukin (IL)-8 and others C-X-C chemokines, are important mediators of leukocyte infiltration and are further discussed in this review article. In addition to these classic chemokines, we also discuss high-mobility group protein-1 (HMGB1), which is a newly identified proinflammatory cytokine in systemic inflammation and may have an important impact on hyperoxic inflammation and lung injury.

IL-8 is an important mediator of acute lung injury and the most potent chemokine and stimulatory factor for neutrophils [115]. Increased levels of IL-8 are detected in the lung during

acute lung inflammation [116,117]. IL-8 produces its chemotactic activity through its interaction with both C-X-C chemokine receptor-1 and -2 (CXCR1 and CXCR2) [118,119]. Several lines of evidence demonstrate that CXCR2 is critical in mediating neutrophil recruitment to the lung during hyperoxic exposure. First, increased expression of CXCR2 was observed in hyperoxic lungs [120]. Following exposure to 80% O₂ for 6 days, CXCR2^{-/-} mice showed reduced neutrophil sequestration, which is associated with significantly decreased lung vascular permeability, edema and injury [121]. Furthermore, administration of neutralizing antibodies and antagonists of CXCR2 ligand, such as cytokine-induced neutrophil chemoattractant-1 (CINC-1), reduced the neutrophil mediated inflammatory responses and markedly increased survival of adult mice subjected to hyperoxia [118,122,123]. Another CXCR2 antagonist, SB265610, blocked chemokine effects on neutrophil viability and lung apoptosis in new born rats exposed to 95% O₂ for 8 days [122]. These studies demonstrate that IL-8 and CXCR2 play critical roles in mediating inflammatory responses under hyperoxic conditions.

As an emerging key player in systemic inflammation, HMGB1 has recently been identified as a lethal mediator of severe sepsis [124]. HMGB1 is a ubiquitous nuclear DNA-binding protein and represents a group of novel intracellular proteins that exhibit chemotactic activities once released into the extracellular milieu [125]. Extracellular HMGB1 has been shown to cause multiple organ failure and contribute to the pathogenesis of diverse disorders including sepsis and acute lung injury [125]. HMGB1 amplifies and extends the inflammatory response by inducing the release of other proinflammatory cytokines from many cell types, including activated macrophages and neutrophils [126]. Elevated levels of HMGB1 are present in the plasma and lung epithelial lining fluids of patients with acute lung injury and in mice exhibiting lung injury-induced by LPS instillation [127]. Intratracheal administration of HMGB1 to mice causes acute lung injury as manifested by neutrophil accumulation, lung edema, and increased pulmonary levels of cytokines, including TNF- α , IL-1 β , and macrophage inflammatory protein-2 (MIP-2) and macrophage migration inhibitory factor (MIF) [128,129]. Therefore, HMGB1 may act as a signaling molecule, inducing both local and systemic inflammatory responses [130,131]. We have investigated whether HMGB1 plays a role in hyperoxic inflammatory responses. Hyperoxia induces the accumulation of HMGB1 in the bronchoalveolar lavage fluid (BALF) in exposed mice and release of HMGB1 from cultured macrophages [132]. Furthermore, addition of recombinant HMGB1 induced the release of TNF- α and MIP-2 under hyperoxic conditions [132]. These results indicate that the HMGB1 may play a pivotal role in mediating hyperoxic-induced lung inflammation and lung injury, and may provide a therapeutic target for treating hyperoxic lung injury, as proposed for other infectious and inflammatory diseases [133].

Transcription factors and proinflammatory cytokines in hyperoxia

Under normal physiological conditions, a highly intricate network of transcription factors regulates the balance of the gene expression of proinflammatory and anti-inflammatory cytokines. However, this balance is perturbed under pathological conditions, resulting in an overwhelming proinflammatory activity [134]. Among the transcription factors involved in modulating proinflammatory responses, NF- κ B is the most important one under hyperoxic conditions, although recent data have also implicated AP-1.

In general, NF- κ B-dependent mechanisms are critical for the enhanced expression of genes encoding inflammatory cytokines, especially chemokines that are important in the recruitment of neutrophils, eosinophils, macrophages, and lymphocytes, acute phase proteins, and immunoreceptors [73,74,135–137]. In hyperoxia, increased NF- κ B activation is one of the first pulmonary responses upon exposure, and this activation occurs before the detection of any proinflammatory cytokine increases [74,138–141]. The NF- κ B-binding motif is present in the

promoter regions of many genes that encode proinflammatory cytokines [81,142–144]. Studies show that the presence of an active NF- κ B site in the IL-8 promoter is essential for hyperoxia-induced IL-8 secretion from U937 macrophages [145]. In addition, NF- κ B also interacts with other sites, such as a NF-IL-6 site in the IL-8 promoter. Such interaction is required for hyperoxic-induced IL-8 gene expression in A549 cells [146]. In addition, hyperoxia can synergistically enhance NF- κ B activation-induced by other cytokines, such as TNF- α and IL-1 β , thus, amplifying the proinflammatory responses [81,147].

The mechanisms modulating NF- κ B activation under hyperoxic conditions involve the activation of I κ B kinase (IKK) pathway. Hyperoxia aggravates IL-1 β -induced activation of IKK, which leads to prolonged degradation of I κ B- α and sustained nuclear translocation and DNA binding of NF- κ B [147]. In addition, exposure of respiratory epithelial cells to 95% O₂ synergistically increased TNF- α -mediated activation of NF- κ B and NF- κ B dependent gene expression by a mechanism involving increased activation of IKK [81]. Furthermore, NF- κ B plays a critical role in the release of HMGB1 into the extracellular matrix, thereby increasing the proinflammatory activity directly and evoking the production of other proinflammatory cytokines [126]. The precise mechanisms that modulate NF- κ B-mediated release of HMGB1 remain unclear because neither the production nor the secretion of HMGB1 is regulated at the transcriptional level [133]. However, the level of NF- κ B activity is closely correlated with the amount of the HMGB1 released extracellularly. In several model systems, including those for endotoxemia and sepsis, suppression of NF- κ B with reagents that exhibit NF- κ B inhibitory ability, such as pyrrolidine dithiocarbamate, ethyl pyruvate or agonists for α 7-nicotinic acetylcholine receptors, effectively controlled HMGB1 release [148,149]. In addition, HMGB1-mediated production of other proinflammatory cytokines involves protein kinases including MAPKs, Akt and NF- κ B [131,148]. Exposure of neutrophils, monocytes, or macrophages to HMGB1 results in increased nuclear translocation of NF- κ B and enhanced expression of proinflammatory cytokines [150]. Unlike LPS, which primarily increases the activity of IKK β , exposure to HMGB1 results in activation of both IKK α and IKK β kinases and scaffolding proteins downstream of Toll like receptor 2 (TLR2) and TLR4 [150]. Taken together, these studies indicate that induction of NF- κ B by hyperoxia is a critical signaling mechanism for evoking an inflammatory response.

Conclusion

In summary, hyperoxia-induced epithelial cell death and leukocyte infiltration play essential roles in the pathogenesis of hyperoxic lung injury. Both events are consequences of many complicated and overlapping signaling pathways, including those that are mediated by MAPKs and redox transcription factors, especially NF- κ B. Hyperoxia-induced damage to pulmonary epithelial cells involves multimodal cell death via both apoptotic and non-apoptotic mechanisms. Despite extensive studies focusing on reducing cell injury and inflammation, no therapeutic interventions have been developed to combat oxidative lung injury resulting from mechanical ventilation with hyperoxia. The dual roles of NF- κ B in modulating both epithelial cell injury/death and pulmonary inflammatory responses make it an obvious target for designing new strategies. Due to the opposing effects of NF- κ B (inhibiting oxidative apoptotic and non-apoptotic epithelial death, versus kindling the release of HMGB1 and production of many proinflammatory cytokines), simple global activation or inhibition of NF- κ B in the lung is not likely to be beneficial. However, modifying NF- κ B activity in specific cell types during specific time windows, such as increasing NF- κ B activation in lung epithelial cells, might be more effective in reducing hyperoxic epithelial damage and inflammatory lung injury. Finally, a better understanding of the overlapping signaling pathways would help to identify new targets to minimize both pulmonary epithelial cell death and proinflammatory responses, and lead to the development of therapeutic strategies for the treatment of oxidative lung injury in ventilated patients.

Acknowledgements

Authors would like to thank Dr. Charles R. Ashby Jr., Thi Le and Nicole Palma for insightful discussion and invaluable suggestions on this manuscript. Authors are supported by grants from ALANYS, NIH, St. John's University College of Pharmacy and the Feinstein Institute for Medical Research at North Shore-Long Island Jewish Health System.

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Abbreviations

ROS	reactive oxygen species
NADPH	nicotinamide adenine dinucleotide phosphate
TUNEL	terminal dUTP nick end labeling
AEC2	type II alveolar epithelial cells
NHBE	normal human bronchial epithelial cells
MLE-12	murine lung epithelial
A549	human alveolar type II cell-like
MAPKs	mitogen activated protein kinases
ERK1/2	extracellular signal regulated kinase
JNK1/2	c-Jun N-terminal protein kinase
AP-1	activator protein-1
mtALDH	mitochondrial aldehyde dehydrogenase
VEGF	Vascular endothelial growth factor
IGF	insulin-like growth factor
KGF	keratinocytes growth factor

HO-1	heme oxygenase 1
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor alpha
IL	interleukine
MEK-1	mitogen activated protein/extracellular signal-related kinase
Nrf2	NF-E2-related transcription factor 2
NF-κB	Nuclear factor-kappa B
STAT	signal transducers and activators of transcription
LPS	lipopolysaccharides
IFN-γ	Interferon gamma
IκB	inhibitor of NF- κ B
IKK	IkappaB α kinase
HMGB-1	high-mobility group protein-1
MnSOD	manganese superoxide dismutase
16HBE	human bronchial cells
MMP	matrix metalloproteinase
ARE	antioxidant response element
PMN	polymorphonuclear
CINC-1	cytokine-induced neutrophil chemoattractant-1
CXCR	

	C-X-C chemokine receptor
MIF	migration inhibitory factor
MIP-2	macrophage inflammatory protein-2
BALF	bronchoalveolar lavage fluid

Fig 1.

A schematic diagram illustrating the NF-κB and other signaling pathways-evoked by exposure to hyperoxia in lung epithelial cells. ROS: reactive oxygen species; NFκB: nuclear factor κB; AOE: antioxidant enzymes; TNF: tumor necrosis factor; IL: interleukin; AP: activator protein; HO: heme oxygenase; MAPK: mitogen-activated protein kinase.