[Frontiers in Bioscience 13, 6653-6661, May 1, 2008]

# Molecular mechanisms of hyperoxia-induced acute lung injury

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## 1. ABSTRACT

Hyperoxia-induced acute lung injury (HALI) is characterized by an influx of inflammatory cells, increased pulmonary permeability, endothelial and epithelial cell death. Reactive oxygen species have been postulated to have a significant role in HALI, in part, by inducing cell death responses regulated by shared common mediators of apoptotic and necrotic pathways. Significant differences exist in the response of the mature and developing lung to HALI.

## 2. INTRODUCTION

Exposure to high concentrations of oxygen is well known to cause significant lung damage. In adult animal models, exposure to hyperoxia leads to pulmonary edema and respiratory failure, progressing to death in 72 to 96 hours (1). Acute pulmonary injury secondary to hyperoxia is complex and multifactorial (2). Hyperoxia-induced acute lung injury (HALI) is characterized by an extensive inflammatory response and destruction of the alveolar-capillary barrier (3-6).

The inflammatory cell influx is orchestrated and amplified by chemotactic factors (7). Morphologic studies in animal models have demonstrated that toxic concentrations of oxygen initially induce focal endothelial cell cytoplasmic swelling and injury and, with continued exposure, necrosis of epithelial cells (8-10). Thus, endothelial and epithelial injury and cell death are major features of HALI (11-14). After acute oxygen exposure, pulmonary microvascular endothelial cells rapidly die, leaving areas of denuded capillary basement membrane (15-16). Disruption of the alveolar-capillary membrane leads to flooding of the alveoli, causing disturbances in pulmonary mechanics and impairment of gas exchange (17-18). Multiple investigators have confirmed the similarities in the stages and morphologic patterns of pulmonary oxygen toxicity in many animal species as well as man (19). Despite these similarities, the responses to HALI in the same animal (murine) model can be very different depending on the genetic background (20-21) and developmental stage (22).

## 3. CELL DEATH MECHANISMS IN HALI

Reactive oxygen species (ROS) have been postulated to have a significant role in HALI and cell death. Studies of this response have led to the free radical theory which suggests that, in 100% $O_2$, lung cells poison themselves by producing an excess of ROS (23-25). In addition, inflammatory cells are also a potent source of ROS. In animal models of HALI, inflammation and lung injury are frequently juxtaposed. This has led to studies investigating the mechanisms of hyperoxia-induced inflammation and the relationship between injury and inflammation in this disorder (26-29). Since HALI is characterized by an influx of inflammatory cells, such cells may represent another important source for ROS (30). Interestingly, inhibitors of the migration of these cells into the lung have been found to create tolerance to HALI (7). In contrast, hyperoxia can induce ALI in animal models that lack leukocytes (31-34) and significant cell death and tissue injury can occur in the absence of a cellular inflammatory response (35). This dissociation demonstrates that HALI-induced tissue injury cannot be attributed solely to local tissue inflammation.

The 2 classical cell death pathways described in HALI lead to necrosis or apoptosis (28, 30, 36). These processes have been considered operationally and mechanistically distinct cell-death responses (37-38). This distinction is probably not true (30). Studies have shown that apoptosis-like deoxyribonucleic acid (DNA) laddering and positive terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining can be seen in cells undergoing necrosis. Inducers of apoptosis can result in necrotic cell death, with both processes being induced by similar agents in similar types of cells. In addition, apoptosis and necrosis can be present concomitantly in tissues experiencing identical forms of injury (39-42). It is possible that both cell death pathways co-exist or that another distinct mechanism may be induced in hyperoxia (30, 43-44). The dose and/or duration of hyperoxia exposure can cause different cell types in the lung to undergo death via distinct or overlapping mechanisms. Recent studies from our laboratory and others have added to this pathogenic paradigm by demonstrating that ROS mediate their effects, in part, by inducing an endothelial and epithelial cell death response with features of apoptosis and necrosis (6, 23-24, 28, 40, 45-46) and that a variety of exogenously administered regulators inhibit these toxic events by regulating local cell death responses (5, 6, 23). In addition, although structural cell apoptosis (such as that seen in HALI) can stimulate tissue inflammation (47-48), hyperoxia-induced inflammation cannot be attributed solely to the nearby cell death response.

Under physiologic conditions, tissue homeostasis is controlled by the tight regulation of apoptosis and necrosis (49-50). These processes are accomplished by the continuous integration of pro- and anti- cell death signals. Activation of key caspases and components of the extrinsic/death receptor and intrinsic/mitochondrial cell death pathways appear to underlie the molecular mechanisms of HALI and cell death. Although cell death can be triggered by a vast array of stimuli and is mediated via an increasingly complex series of pathways, the vast majority of cell death signals engage the cell death machinery at the level of the cell membrane or the mitochondria. The membrane (extrinsic) pathway triggers surface "death receptors" such as Fas, which binds Fas ligand, and tumor necrosis factor receptor 1 (TNFR1), which binds TNF and lymphotoxin, which activate caspase-8. Other key regulators include the many members of the Bcl-2 gene family (which can be divided into 3 groups: antiapoptotic Bcl-2 and Bcl-$x_L$, proapoptotic Bax-type proteins, and proapoptotic BH3-domain-only family members), protein kinase B/Akt, and the redox-sensitive transcription factor NF-κB (23, 30). In the intrinsic response, mitochondrial dysfunction signals cell death. In this response, BH3 domain-only family members such as Bid are activated and interact with Bax-type proteins (Bax, Bak, Bok) to form or interact with mitochondrial pores, release cytochrome *c*, activate caspase-9 and induce cell death (23, 51-53). The mitochondrial-dependent pathway is probably more relevant in HALI, as mitochondria maintain the cellular levels of adenosine triphosphate (ATP) and are able to release death-promoting factors, such as cytochrome *c* (30).

## *4. HALI: IN VITRO* STUDIES

### 4.1 Endothelial cells

In human pulmonary artery endothelial cells (HPAE), the threshold for toxicity appeared to be an oxygen concentration of ~60% (54). On exposure to hyperoxia, endothelial cells generate ROS (55, 56) that may result in cell injury or be exposed to ROS generated by neutrophils (57). One of the mechanisms of ROS generation in HPAEs is via the regulation of the MAP kinase (MAPK) pathway (58). In bovine lung microvessel endothelial cells (BLMVECs), hyperoxia attenuated phorbol-ester induced protein tyrosine phosphorylation and phospholipase D activation (59). Interestingly, enhancement of hypoxia-inducible factors 1 and 2alpha could be achieved by blocking prolyl 4- hydroxylase in human lung microvascular endothelial cells (HLMVEC) exposed to hyperoxia (60). Release of ATP from HLMVEC when exposed to hyperoxia led to activation of ERK 1/2, PI3K and mTOR, primarily through activation of purinoceptors $P_2Y_2$ and $P_2Y_6$ (61). Activation of ERK and Akt (62) have been found to protect HLMVEC from hyperoxia-induced cell death. BLMVECs that overexpressed manganese superoxide dismutase (MnSOD) showed a significant resistance to hyperoxia (63). Recent studies have shown that heme oxygenase-1 and carbon monoxide confer protection from hyperoxia-induced murine lung endothelial cells (MLEC) death mediated via extrinsic and intrinsic apoptotic pathways and STAT-3 (64-66).

### 4.2 Epithelial cells

Molecular mechanisms of hyperoxia-induced death in lung epithelial cells have recently been reviewed (67-69). Lung epithelial cells (A549 and MLE-12) die by necrosis on exposure to hyperoxia (41, 46, 70). Figure 1 shows a possible sequence of events following hyperoxia.

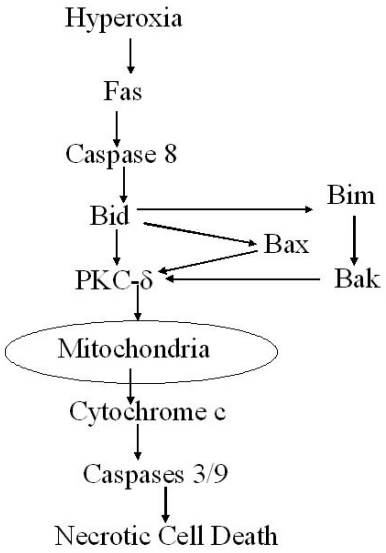


**Figure 1.** Molecular mechanisms of hyperoxia-induced necrotic cell death.

Hyperoxia exposure to the lung activates the cell death receptor, Fas, (a member of the TNFR family) with resultant activation of caspase 8. This initiates a cascade of events with activation of Bax, Bid, Bim, and Bak. This is followed by increased expression of PKC-δ, which moves into the mitochondria, stimulating the release of mitochondrial cytochrome *c*. This, in turn, results in cleavage of effector caspases 3 and 9, culminating in necrotic cell death. Proof of this concept has been shown in multiple studies (46, 70-72). In MLE-12 cells, hyperoxia exposure resulted in activation of the transcription factor AP-1 and MAPK, p38 and JNK (44). In MLE-12 cells, inhibition of the JNK pathway improved cell viability (73). ERK-dependent pathways were found to be protective (74) or enhanced (75) hyperoxia-induced cell death in *in vitro* studies. Other transcription factors induced by hyperoxia in epithelial cells include NF-κB, STAT and Nrf2 (68). NF-κB has been shown to be protective against hyperoxia-induced cell death in multiple cell culture studies (68).

## 5. *HALI: IN VIVO* STUDIES

The initial response of the lung to hyperoxia is damage to the microvascular endothelial cells. This is usually accompanied by an inflammatory cell exudate and release of soluble mediators of inflammation, which include cytokines (7). As mentioned earlier, while inflammatory cells may contribute, their presence is not essential for HALI (19, 35, 67). Destruction of the pulmonary capillary endothelium occurs next. This is followed by death of the Type I pneumocytes. Disruption of the alveolar-capillary unit causes pulmonary edema. The role of Na,K-ATPase, which was localized to the alveolar epithelium, in contributing to lung edema clearance has recently been described (76). The Type II pneumocytes are more tolerant to hyperoxia, though evidence of DNA damage to these cells, without loss of their viability, has been reported (77).

Antioxidant activity in the glutathione pathway may be modulated by thioredoxin-related mechanisms and impact on HALI (78). N-acetylcysteine (NAC), a precursor of glutathione (GSH), which functions as an antioxidant, induced the overexpression of MnSOD mRNA and protein, and decreased HALI, but did not alter the expressions of other antioxidant enzymes, including CuZnSOD, extracellular SOD, and glutathione peroxydase 1 (79). A recent review, however, suggests that the role of MnSOD, CuZnSOD, or cellular glutathione peroxidase in preventing HALI is negligible, and other cellular antioxidant enzymes and systems may be primarily used by the lungs in the defense against hyperoxia (80). NAD (P)H/NRH:quinone oxidoreductases (NQO1 and NQO2) double null mutant mice demonstrated significant degree of HALI, compared with wild-type mice (81). Mice deficient in glutaredoxin 1 (Grx1) did not show evidence of more HALI, compared to wild type controls (82).

Mice with a targeted disruption of the transcription factor, Nrf2, had suppressed expression of several antioxidant response element bearing anti-oxidant/detoxifying enzyme genes and their activities after exposure to hyperoxia (83). Mice lacking the transcription factor Nrf2 had increased HALI and mortality (83). Micoarray analyses of the genes that may be involved in this pathway have been reported (84). Regarding the role of nitric oxide (NO), most of the data from cell culture systems and adult animal models of HALI suggests that endogenous NO has a protective role (85), though controversy exists (86-87).

Tolerance to HALI was noted in hypotransferrinemic mice. However, this protective effect was not accompanied by any increase in the levels of intracellular antioxidants, inflammatory cytokines, and heme oxygenase-1. Interestingly, there were elevated expressions of ferritin and lactoferrin in the lungs of hypotransferrinemic mice, especially in the alveolar macrophages (88).

Hyperoxia caused significant increases in pulmonary and hepatic CYP1A1 activities in Ah-receptor (AHR) sufficient, but not in AHR null mutant mice. In contrast, hyperoxia augmented hepatic CYP1A2 expression in both wild-type and AHR null mutant mice, which suggested that AHR-independent mechanisms contributed to the CYP1A2 regulation by hyperoxia. AHR null mutant mice exposed to hyperoxia had evidence of increased HALI (89).

Overexpression of STAT3C, a constitutive active form of STAT3, protected against HALI (27). This protection was mediated at least partially through inhibition of hyperoxia-induced synthesis and release of matrix metalloproteinase (MMP)-9 and MMP-12 by neutrophils and alveolar resident cells (27).

In contrast to the *in vitro* studies (73), JNK 1 and 2 null mutant mice lungs had increased TUNEL positive staining on exposure to hyperoxia (90).

We (35, 70) and others (67) have implicated the constituents of the extrinsic and intrinsic cell death pathways in HALI. These include members of the death receptor superfamily (Fas, Fas-L, and TNFR1) (35, 70, 91-92) and Bcl family members (Bax, Bak, Bid, Bim, Bcl-$X_L$, Bfl-1/A1) (23, 35, 67, 70). While Bid null mutant mice were protected, Fas, Fas-L, and TNFR1/2 null mutant mice were not protected (46), and A1 null mutant mice displayed a increased mortality (23), on exposure to hyperoxia. In addition, hyperoxia can induce other cell death related molecules such as p53 and p21 (67). Hyperoxia exposure to p21 null mutant mice led to increased mortality (93), in contrast to absence of effect in p53 null mutant mice (67). Toll-like receptor 4 (TLR4) null mutant mice had increased susceptibility to HALI, which correlated with their inability to upregulate Bcl-2, Akt and heme oxygenase-1 (94).

Leukotriene inhibitors did not appear to significantly impact on HALI in 3-week old rat pups exposed to hyperoxia for 1 week (95). Leptin induced, and resistance to leptin attenuated, HALI and decreased pro-inflammatory cytokines (96). Absence of the chemokine receptor, CXCR2 in mice led to decreased HALI and increased survival (97). IFN-gamma null mutant mice had decreased HALI, but only in the early phase (98). High mobility group protein-1 (HMGB1), a DNA-binding protein that belongs to a group of intracellular proteins that show chemotactic properties once released extracellularly may have a role in HALI (68).

Specific cytokines (and growth factors) appear to ameliorate HALI by affecting cell death pathways. These include GM-CSF, KGF, IL-11, IL-13, and VEGF. Details can be found in a recent review (7). In addition, we have reported that lack of the angiogenic cytokine, Angiopoiretin 2, is protective against HALI, necrotic cell death and increased survival (70). Importantly, we found Angiopoietin 2-mediated hyperoxia-induced increases in initiator and effector caspases, namely caspase-3, -8 and -9 (70). We also noted Angiopoietin 2-dependent modulation of the mediators of cell death pathways in hyperoxia, namely Fas, Bax, Bak, Bim and Bid (70). Thus, hyperoxia activated the extrinsic and mitochondrial cell death pathways, critical initiator and effector caspases, and BH3 only proteins via mechanisms that were Angiopoietin 2-dependent.

## 6. DEVELOPMENTAL DIFFERENCES IN HALI

Newborn (NB) animals, in many species, have been known to be more resistant to HALI than their adult counterparts (99). Adult mice exposed to 100% $O_2$ usually die in 3-5 days while similarly exposed NB mice die by 7-10 days.

In a study evaluating cell-specific alterations in mRNA expression of surfactant protein-A, tissue inhibitior of metalloproteinases, and metallothionein, on exposure to hyperoxia, revealed that adults respond faster and to a greater extent than newborns (100). In neonatal rats exposed to hyperoxia, DNA damage was evident at 3 days only, mostly in epithelial cells, along with a increased expression of Bax (101). The mechanisms and cell types involved, contributing to increased neonatal tolerance to hyperoxia are unknown. Keeney *et al* (102) studied the effect of *in vivo* exposure to 85% $O_2$ for 72h on the activities of the antioxidant enzymes (AOE), glutathione peroxidase (GPX), catalase (CAT) and SOD, in alveolar type II pneumocytes (TIIP) and whole lung from adult and neonatal rats. Baseline AOE activities were generally lower in neonatal TIIP compared with adults. Baseline enzyme activities did not differ in neonatal TIIP and lung homogenates except for lower CAT activity in TIIP. Hyperoxic exposure resulted in 35-38% increases in AOE activities in neonatal whole lung. In neonatal TIIP, SOD activity increased by 170% after hyperoxia, whereas CAT and GPX were not significantly changed. In the adult whole lung, hyperoxic exposure resulted in increases in only GPX activity, whereas in adult TIIP there was a significant decrease in SOD activity after $O_2$ exposure. Therefore, although baseline AOE activities were not higher in neonatal TIIP compared with whole lung, there were differences in the AOE responses of adult and neonatal TIIP to hyperoxia, particularly with respect to SOD. The ability of the neonatal TIIP to respond to hyperoxia with an early increase in SOD activity may contribute to the enhanced $O_2$ tolerance of the neonate.

We have measured the activities of CAT, glutathione reductase, GPX, and cytosolic SOD in cultures of adult and fetal (19-day, term is 21 days) rat TIIP exposed to 95 $O_2$ for 24h (103). Hyperoxia exposure resulted in 53 % viability in the adult TIIP with a significant threefold increase in the activities of all the AOE. The fetal TIIP were more resistant to hyperoxia (99 % viability). However, in the fetal TIIP, only SOD and GPX levels were significantly increased (4-fold and 2.3-fold, respectively) compared with fetal controls. We concluded that fetal TIIP are more resistant to hyperoxia than adult TIIP in terms of viability; other protective antioxidant factors might account for the better survival of TIIP in hyperoxia (103). Interestingly, extra cellular SOD (EC-SOD) protein secretion increased, but EC-SOD enzyme activity did not change with hyperoxia exposure in neonatal rat lungs (104).

In the NB animal, endogenous NO appeared to be harmful, had no effect or was protective in hyperoxia-induced lung injury. The data are conflicting on the issue of whether exogenous NO is protective or damaging in the presence of hyperoxia on lung cells and animal models (85, 105-107)

Hyperoxic exposure to the adult and NB lung had variable effects on the expression of interleukin (IL)-1alpha and IL-1beta (7). Increased IL-6 levels were seen in adult lungs by day 3 and in the NB lungs by day 10 of exposure to hyperoxia. IL-8 also peaked around day 10 in the NB lung but there were no significant changes in IL-10 (22). Recently, we have reported that IL-6 overexpression led to significantly increased mortality and HALI in neonatal mice, in sharp contrast to increased survival in adult mice (108).

Monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-2, and interferon gamma-induced protein (IP)-10 mRNAs were present in whole NB lung by 4 days of hyperoxia and were markedly elevated by 7 days (109). Levels of mRNA for MCP-1, MIP-1 alpha, and MIP-2 were elevated to a lesser extent by 72h of hyperoxia in adults. MCP-1 mRNA abundance was moderately elevated in scattered areas of perivascular tissue, peribronchiolar tissue, and the alveolar interstitium in 4-day hyperoxic NB and markedly upregulated diffusely throughout the peripheral airspaces in 7-day hyperoxic NB. MCP-1 mRNA abundance was limited to scattered perivascular areas and airspaces in 72h hyperoxic adults. These differences in the intensity, timing, and distribution of chemokine mRNA abundance between adult and NB mice may help to explain the marked differences in their susceptibility to HALI (109). A chemical antagonist of cytokine-induced neutrophil chemoattractant -1 (CINC-1, which acts via CXCR2) was able to prevent neutrophil accumulation and reduce DNA damage in neonatal rat lungs exposed to hyperoxia (110-111).

A common feature of hyperoxic gene regulation is involvement of activator protein (AP)-1. Yang *et al* evaluated lung AP-1 binding as well as that of the AP-1 subunit proteins c-Fos, c-Jun, phosphorylated c-Jun, Jun B, and Jun D after exposure to >95% $O_2$ for 3 days (112). Unlike adults, neonates showed no increased AP-1 binding in hyperoxia despite a high affinity of the AP-1 binding complexes for phosphorylated c-Jun and Jun D as demonstrated by supershift of these antibodies with the AP-1 complexes. Moreover, neonatal lungs exhibited two distinguishable AP-1 binding complexes, whereas adult lungs had one AP-1 binding complex. In neonates, sequential immunoprecipitation revealed that the lower AP-1 complex was composed of proteins from both the Fos and Jun families, whereas the upper complex consisted of Jun family proteins, with predominance of Jun D. In adults, the single AP-1 complex appeared to involve other Fos or non-Fos or non-Jun family proteins as well. Neonatal lungs showed a higher level of Jun B and Jun D immunoreactive proteins in both air and hyperoxia compared with those in adult lungs. These results suggest that significant maturational differences in lung AP-1 complexes exist and these may explain transcriptional differences in hyperoxic gene regulation (112).

Differences in lung heme oxygenase-1 (HO-1) regulation have been demonstrated in NB rats after exposure to hyperoxia. Contrary to adults, neonates do not demonstrate increased lung HO-1 induction nor AP-1 binding in hyperoxia (113). As AP-1 activation can be post-translationally modified by oxidants or reductants, Yang *et al* investigated whether differences in lung glutathione (GSH) content could account for the maturational differences in AP-1 activation and subsequent HO-1 gene regulation after hyperoxia. Changes in lung GSSG/GSH ratio did not alter AP-1 binding but did increase HO-1 mRNA in neonates. These data suggest that the neonatal lung is relatively resistant to AP-1 activation and HO-1 induction by GSH perturbation (113).

Differences in NF-κB activation in neonatal mice, compared to adult mice, might account for their increased survival and decreased HALI (114).

The differences in the type and timing of release of various interleukins (7, 22), chemokines (7, 109, 115), AP-1 (112), HO-1 (113), NF-κB (114), and AOE (102-103) in the developing lung may account for the NB animals tolerance to hyperoxia. It is thus quite obvious that significant differences exist in the response of the mature and developing lung to HALI. The unique pathogenesis of HALI in the NB is still essentially unknown (7, 22).

## 7. CONCLUSIONS

An important caveat to remember is that while cell cultures, *in vitro* and *in vivo* animal models are useful to understanding the biological mechanism (s), they may not necessarily reflect what goes on in the human body. In addition, since extrapolation of data from adults to NB is fraught with many missteps (22, 108) (and doing so in humans has been shown many times to be life threatening), it is essential to use developmentally-appropriate *in vitro* and *in vivo* models to delineate their unique responses. This has major translational ramifications. One relevant example is HALI in the human adult does not lead to bronchopulmonary dysplasia (BPD), which is specific to HALI occurring in the developing lung (116). Understanding the mechanism (s) of HALI in the developing lung will potentially lead to improved understanding even in the adult lung, as key developmental genes change during HALI in adult mice (117). Such an approach would have the maximum potential to impact on translation to the relevant human disease in the context of HALI.

## 8. ACKNOWLEDGEMENT

Supported in part by Grants ATS-07-005 from the American Thoracic Society, 0755843T from the American Heart Association and HL-74195 from the NHLBI of the National Institutes of Health, USA

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**Key Words:** Oxygen, Pulmonary, Cell death, Apoptosis, Necrosis, Review

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