DNA damage and cell cycle checkpoints in hyperoxic lung injury: braking to facilitate repair

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O’Reilly, Michael A. DNA damage and cell cycle checkpoints in hyperoxic lung injury: braking to facilitate repair. Am J Physiol Lung Cell Mol Physiol 281: L291–L305, 2001.—The beneficial use of supplemental oxygen therapies to increase arterial blood oxygen levels and reduce tissue hypoxia is offset by the knowledge that it injures and kills cells, resulting in increased morbidity and mortality. Although many studies have focused on understanding how hyperoxia kills cells, recent findings reveal that it also inhibits proliferation through activation of cell cycle checkpoints rather than through overt cytotoxicity. Cell cycle checkpoints are thought to be protective because they allow additional time for injured cells to repair damaged DNA and other essential molecules. During recovery in room air, the lung undergoes a burst of proliferation to replace injured and dead cells. Failure to terminate this proliferation has been associated with fibrosis. These observations suggest that growth-suppressive signals, which inhibit proliferation of injured cells and terminate proliferation when tissue repair has been completed, may play an important role in the pulmonary response to hyperoxia. Because DNA replication is coupled with DNA repair, activation of cell cycle checkpoints during hyperoxia may be a mechanism by which cells protect themselves from oxidant genotoxic stress. This review examines the effect of hyperoxia on DNA integrity, pulmonary cell proliferation, and cell cycle checkpoints activated by DNA damage.

deoxyribonucleic acid; genotoxic stress; phosphorylation; proliferation; p53

since their evolution 500–1,500 million years ago, aerobic cells have been paying a price for their ability to obtain more energy from carbon sources compared with anaerobic cells because cytotoxic reactive oxygen species (ROS) form during aerobic respiration that can damage DNA, lipids and proteins. These ROS include superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH$^-$), and peroxynitrite (ONOO$^-$). Because ROS are formed during normal respiration, cells have developed enzymatic and nonenzymatic antioxidant defense systems to reduce intracellular redox levels. The enzymatic antioxidant systems include superoxide dismutases, catalase, and glutathione peroxidase. Nonenzymatic systems include vitamins (A, E, and C), selenium, and other nutritional molecules. These systems usually provide adequate protection against the damaging effects of normal oxygen metabolites (see Ref. 97 for a review). Oxidative stress and damage occur when the balance of produced ROS exceeds the capacity of the cell to detoxify.

A second line of defense against oxidant injury involves repair of damaged molecules important for cellular function. Unfortunately, identifying enzymes that repair oxidant damage has been difficult because ROS can attack all molecules and organelles, resulting in cell injury and death. Several excellent reviews (92, 96, 139) have been written recently that focus on ROS and various signal transduction pathways activated in response to oxidative stress. These reviews, however, did not address in detail the cellular response to oxidant DNA damage, which occurs when cells are injured.
by ionizing radiation (IR), bleomycin, prooxidant particles, hydrogen peroxide, hyperoxia, or any other chemical that oxidizes cells (13, 24, 73). Radical attacks on DNA produce nearly 100 lesions that include oxidation of bases and sugars, depurination, depyrimidation, and phosphodiester single- and double-strand breaks (42). ROS can also oxidize lipids, which, in turn, damage DNA and contribute to oxygen toxicity (23, 138). Although molecular oxygen is inert when exposed directly to DNA (55), its genotoxic effects on cells have been known for nearly 50 years (34). Because hyperoxia produces free radicals (164), many investigators use hydrogen peroxide as a surrogate for hyperoxia in their studies. Even though hydrogen peroxide oxidizes DNA, it remains questionable whether it exerts the same damage as hyperoxia. For example, the genotoxic effects of hydrogen peroxide include mutagenic single-strand breaks, whereas hyperoxia induces chromosome aberrations and sister chromatid exchanges (56, 57, 59). Second, iron chelators such as desferrioxamine have no effect on hyperoxia-induced clonogenic survival, whereas they are protective to cells damaged by hydrogen peroxide (58). It also takes several days for oxygen to injure and kill cells, whereas hydrogen peroxide takes immediate effect and has an extremely short half-life. Thus most studies with hydrogen peroxide are likely studying repair. In contrast, studies with hyperoxia are done under continuous exposure where repair is compromised by continual damage. Even with these differences, ROS such as those produced by hyperoxia or hydrogen peroxide clearly damage genomic DNA.

Cells can repair DNA damage, fixate the mutation in their genome, or die by apoptosis or necrosis. Because apoptosis is a swift and noninflammatory form of cell death compared with necrosis, one could argue that it is a beneficial method to deal with cells injured by ROS (121). However, it is also advantageous for cells, especially stem cells such as alveolar type II cells, to undergo repair so that the tissue and organism are not compromised. Given that nearly 10,000 nucleotides/nucleus are oxidized each day during normal respiration (101), one must consider that DNA repair is as important in the cellular response to superphysiological levels of oxygen as programmed cell death. This paper discusses the genotoxic effect of oxygen on cell proliferation, with particular emphasis on the lung, and concludes with a review of how cells sense DNA damage and transduce this signal to growth-suppressive molecules.

HYPEROXIA AND ITS EFFECTS ON PROLIFERATION

Cell proliferation during hyperoxic exposure. Detailed morphometric and ultrastructural studies in animal models have been used over the past 30 years to understand how hyperoxia affects cells. Adult rats, mice, and monkeys exposed to lethal levels of oxygen (>90%) appear unaffected for the first 48 h, after which they become lethargic and fail to eat or drink, with mortality between the fourth and seventh days (2, 16, 80). One of the first morphological signs of injury is focal cytoplasmic swelling of microvascular endothelial cells and interstitial edema that is followed by endothelial cell fragmentation. In contrast, the alveolar epithelium is relatively unaffected until after 72 h of exposure when cytoplasmic swelling and death of the type I epithelial cell occur. Closer examination revealed that the death of endothelial and type I epithelial cells was due to progressive swelling of cytoplasm, nuclei, mitochondria, and cisternae of the endoplasmic reticulum that was followed by disintegration of the plasma membrane. These morphological signs of death are consistent with death by necrosis (121). Although hyperoxia initially injured and killed endothelial cells, survival was dependent on maintaining the structural integrity of the type I epithelial cell (2, 37). Thus hyperoxia initially injures and kills endothelial cells followed by type I epithelial cells, with less of an effect on type II cells.

The effect of hyperoxia on proliferation in the adult lung is a relatively understudied area of research, probably because the mitotic index is very low. Mitotic labeling studies with [3H]thymidine in adult mice revealed that lymphocytes are the most proliferative cell type within the lung, albeit with only a 2% labeling index (17, 47). Microvascular endothelial cells are the second most proliferative population, with type II epithelial cells and macrophages representing a minor percentage. Similar results were found in 3-wk-old rats where the mitotic index of type II cells was estimated at 0.25% (83). One of the first studies on the toxic effects of oxygen in the rodent lung found that microvascular endothelial cells were injured at sublethal levels of oxygen [fraction of inspired oxygen (FiO₂) <90%], whereas epithelial cells appeared unaffected (48). Based on this finding and the knowledge that endothelial cells had a higher mitotic index, Evans and Hackney (48) argued that they would be more susceptible than epithelial cells to inhaled toxic pollutants. Crapo et al. (37) reported similar findings in their studies with rats exposed to FiO₂ of 85%, which killed endothelial cells without injuring epithelial cells. However, this concentration of oxygen stimulated type II epithelial cells to undergo one round of proliferation. Although higher concentrations of oxygen typically inhibit proliferation and kill both endothelial and type I cells (49), one study found that they also promoted ex vivo proliferation of fibroblasts isolated from hyperoxic rats (85). Because sublethal levels (<85%) of oxygen generally stimulate proliferation while lethal levels (>90%) inhibit it, growth cessation may be a response to cellular damage.

In contrast to the adult lung, the mitotic index of the postnatal lung is significantly higher. At birth, rat lung fibroblasts and microvascular endothelial cells have the highest labeling index (83). Over the first few days of life, the labeling index of fibroblasts increases from 6.5 to 14% as the alveoli septate. Thereafter, proliferation of fibroblasts and endothelial cells decreases. Type II epithelial cells have the lowest mitotic index at birth, but it increases over the first week of life to ~7%
before decreasing to the low level seen in the adult lung. During this time, the type I cell population increases without significant label retention. This was interpreted as evidence that type I cells originate from type II cells (5). Like the adult lung, a $F_{O_2} > 95\%$ inhibits proliferation of the newborn lung. However, newborns are more resistant to the toxic effects of oxygen, perhaps due to their greater level of antioxidant enzymes (33, 53, 160). Although proliferation initially decreases in newborns exposed to hyperoxia, it resumes after several days even when continuously exposed (15, 104, 151). In fact, the mitotic index increases significantly, as if the lung was trying to “catch up” for the delay caused by the initial exposure. The use of exogenous surfactant and steroids for the treatment of respiratory distress and prevention of bronchopulmonary dysplasia has revealed additional problems associated with lung prematurity that have led some to suggest that it involves an arrest in alveolar and vascular development (78). It remains unclear how much of this is due to prematurity or lung injury caused by infection, supplemental oxygen, and/or assisted ventilation. Thus the identification of molecules that regulate pulmonary cell proliferation could provide insight into these important questions.

A major question regarding the effects of oxygen on proliferation is whether they reflect overt cytotoxicity or activation of specific cell cycle checkpoints. If hyperoxia inhibited proliferation through general toxicity, the cells would be expected to cease proliferation when the rate-limiting molecules required for proliferation are compromised. Several studies (11, 129, 130) have provided supportive evidence that the inactivation of mitochondrial enzymes, mitochondrial damage, and gradual respiratory failure occur in HeLa and Chinese hamster ovary (CHO) cell lines and intact lungs in response to hyperoxia. Hyperoxia also decreased ATP levels, consistent with the loss of mitochondrial function (7, 60). Although these findings suggest that changes in cell proliferation may be due to overt cytotoxicity, other studies have discovered that hyperoxia activates a $G_1$ cell cycle checkpoint (36, 122, 132).

Cell cycle checkpoints as defined by Hartwell and Weinert (69) represent specific events during the cell cycle where cells cease proliferation to assess whether replication should continue. These gaps in the cell cycle were identified by a number of assays, including fusion of nuclei isolated from cells in various stages of cell division. Cell replication can be divided into two stages: the M phase where cell division occurs and the interphase, the time between M phases (Fig. 1). The interphase consists of the $S$ phase where DNA replication occurs and two gap phases where cell cycle progression temporarily ceases. The first gap phase, $G_1$, is the interval between the $M$ and $S$ phases. It is during this time that cells presumably decide whether conditions are favorable for replication. The second interval, $G_2$, separates the $S$ phase and the $M$ phase. Here cells could ensure that DNA had been faithfully replicated before cytokinesis occurred. Movement through the cell cycle is regulated by kinases that have catalytic part-

![Diagram](http://ajplung.physiology.org/)

**Fig. 1.** Overview of the cell cycle depicting separation between interphase and mitosis ($M$). Interphase can be further divided into $G_1$, $S$, and $G_2$ phases. Each phase is regulated by the activities of cyclin-dependent kinases (Cdk) that bind catalytic cyclin proteins (A, B, D, and E). DNA damage inhibits progression at $G_1$, $S$, and $G_2$ phases through unique molecular signals.

ners called cyclins because their expression cycles during the cell cycle (see Ref. 45 for a review). Early $G_1$ cyclins include cyclins D1, D2, and D3 that complex with cyclin-dependent kinases (Cdk) 4 or 6. Cyclin E is expressed in late $G_1$ and binds Cdk2. The $S$ phase is regulated by cyclin A interacting with Cdk2, and the $G_2$ phase is regulated by cyclin B associating with cdc2 (also called Cdk1). DNA replication occurs when the $G_1$ and $S$ phase kinases phosphorylate the retinoblastoma (Rb) gene product, resulting in release of the transcription factor E2F that increases the transcription of genes like thymidine kinase that are required for DNA synthesis. This is an oversimplification because there are two other proteins (p107 and p130) that are related to Rb and six E2F (E2F-1 through E2F-6) proteins. DNA damage inhibits proliferation by activating signal transduction pathways that block the $G_1$, $S$, and $G_2$ phases of the cell cycle. For example, DNA damage increases expression of the tumor suppressor p53, which inhibits proliferation in $G_1$ by increasing transcription of the $G_1$ Cdk inhibitor p21$^{Cip1/WAF1/Sdi1}$ (hereafter p21) (44, 116, 146). This pathway is discussed in more detail later.

If hyperoxia were activating cell cycle checkpoints, one would expect to see changes in the expression of molecules that regulate cell proliferation. And, in fact, hyperoxia does alter the expression of mitogens and antimitogens. For example, the mitogen basic fibroblast growth factor and insulin-like growth factor I increased in adult rat lungs exposed to 85% oxygen (19, 66). As shown in a newborn rabbit model of oxygen injury, hyperoxia increased expression of keratinocyte growth factor, which stimulates proliferation of epithelial cells, principally type II cells (27, 142). Sublethal levels of oxygen in rats stimulated type II cell proliferation and decreased expression of parathyroid hormone, which inhibits type II cell proliferation (70). Because lethal levels of oxygen inhibit proliferation, one would predict that they also induce growth-inhibi-
inhibits proliferation at the G1/S boundary. Using TGF-β, which is a potent inhibitor of epithelial cell proliferation (21, 99, 106), TGF-β expression decreased on recovery in room air when proliferation occurs. Similarly, hyperoxia increased expression of p21 in adult and newborn mice (95, 109). Like TGF-β, p21 expression also decreased during recovery in room air. Perhaps the most compelling argument for activation of cell cycle checkpoints is the recent observation that hyperoxia did not inhibit proliferation in p21-deficient mice even though it still caused morbidity and mortality (111).

Several in vitro cell line models have also been used to understand how hyperoxia alters cell proliferation at the molecular level. Changes in cell proliferation are one of the earliest signs that hyperoxia has affected cell function and were first described in HeLa cells by Rueckert and Mueller (128) in 1960. The toxic effects of oxygen were not due to secretion of cell poisons because medium collected from oxygen-exposed cultures did not kill normoxic cells (137). Using a rat type II epithelial cell line that was immortalized with SV40, Clement et al. (32) showed that 95% oxygen inhibited proliferation and decreased translation of histone and thymidine kinase mRNAs, which are required for DNA replication. Subsequent studies revealed that hyperoxia also increased mRNA levels of the cytokine TGF-β and its receptors, consistent with in vivo studies in rodents (26, 36). It also increased expression of p21, which inhibited G1 cyclin E-dependent kinase activity, consistent with activation of a G1 checkpoint that prevented DNA replication. Based on the finding that neutralizing antibodies to TGF-β increased cyclin E-dependent kinase activity, the authors concluded that hyperoxia inhibits proliferation in G1 through p21 that was induced by TGF-β. However, this concept was not uniformly held in other in vitro models because Rancourt et al. (123) demonstrated that hyperoxia does not inhibit proliferation of Mv1Lu mink lung adenocarcinoma cells through TGF-β signaling. In this model, Mv1Lu cells were shown to growth arrest predominantly in the S phase of the cell cycle, whereas TGF-β inhibits proliferation at the G1/S boundary. Using A549 lung adenocarcinoma and HCT116 colon carcinoma epithelial cells that lacked p21, Rancourt et al. (122) further showed that hyperoxia inhibits proliferation in the G1 and S phases of the cell cycle, whereas p21 mediates the G1 arrest. The finding that Mv1Lu cells arrested in the S phase and not in the G1 was due to the inability of hyperoxia to induce p21 in these cells. An additional study (132) revealed that hyperoxia inhibited proliferation of bronchial smooth muscle airway cells in the G1 and S phases along with induction of p21. A similar study (14) was performed in the human breast carcinoma cell line T47D-H3. Approximately 40% of these cells accumulated in the S phase when exposed to hyperoxia, without p21 binding to the G1 and S phase Cdk2. Although this study differed from other studies in that hyperoxia did not increase p21 protein levels, it confirmed that hyperoxia induces a p21-independent S phase arrest. In summary, these studies all show that hyperoxia inhibits proliferation through a p21-dependent G1 arrest and a presently uncharacterized S phase arrest. Because little is known about S phase checkpoints, it will be interesting to discern whether growth arrest in the S phase is mediated by activation of a novel checkpoint or overt cytotoxicity.

Cell proliferation during recovery in room air. Normal repair during recovery in room air is dependent on the rapid and balanced proliferation of fibroblasts and endothelial and epithelial cells (1, 141). In addition to terminating growth-inhibitory signals like TGF-β and p21, growth-stimulatory signals are likely to be expressed because proliferation exceeds the basal level typically observed before exposure. Pulse-chase labeling studies (1, 80) with [3H]thymidine in mice and monkeys showed that type II epithelial cells proliferate and differentiate into type I cells. Although it remains to be determined how endothelial cells are replaced, proliferating type II cells isolated from adult rabbits were found to transiently express high levels of the potent endothelial cell mitogen vascular endothelial growth factor (91). Thus type II cells play a major role in the normal homeostasis and repair of the lung because they express pulmonary surfactant, act as stem cells for type I cells, and may participate in proliferation of endothelial cells by expressing vascular endothelial growth factor. In addition, mitogens that are expressed during exposure, like basic fibroblast growth factor, keratinocyte growth factor, and insulin-like growth factor I, could potentially stimulate proliferation during recovery when growth-inhibitory signals decrease (19, 27, 31). Fibroblasts also produce presently unidentified growth-stimulatory molecules for alveolar type II cells exposed to 100% oxygen (50). Although it remains unknown whether these mitogens affect cell proliferation during recovery, increased G1 and S phase cyclin kinase activity has been reported in recovering rat lungs, consistent with reactivation of the cell cycle machinery (22).

An imbalance between proliferation of type II cells and fibroblasts can lead to chronic inflammation and fibrosis. Although inflammation and collagen production are likely to be important during the remodeling phases, failure to terminate these processes can be catastrophic. This is most evident in the bleomycin-induced model of pulmonary fibrosis where continued proliferation of type II cells is observed (3). Modest septal thickening and increased interstitial collagen have been reported in lungs recovering from lethal levels of oxygen or in lungs exposed for long periods of time to sublethal levels, consistent with modest fibrosis (4, 99, 151). One study (4) using an explant model to recapitulate fibrosis after hyperoxic injury observed that fibroblast proliferation exceeded epithelial cell proliferation due to epithelial cell necrosis. However, attenuation of fibroblast proliferation with proline analogs to block collagen production failed to inhibit fi-
brosis (18). This study also discovered that normal repair involves interactions between fibroblasts and the epithelium because epithelial cell proliferation was affected by the drugs too. Another study (20) found that cultured type II cells isolated from rat lungs exposed to hyperoxia undergo apoptosis even though they are thought to be resistant to oxidant damage. In summary, injured and dead cells are replaced during recovery through increased proliferation and differentiation. Normal repair is dependent on the balanced proliferation of both epithelial and mesenchymal cells. Given that controlled proliferation is beneficial during and after oxidant injury, let us examine how cell cycle progression is regulated.

CELL CYCLE AND CHECKPOINTS

G1 checkpoints. As described in Cell proliferation during hyperoxic exposure, the cell cycle can be divided into discrete time periods where G1 represents the first phase of interphase. During this time, Hartwell and Weinert (69) argued, cells could ensure that the conditions for DNA replication were favorable. Cyclin D and E kinases phosphorylate the Rb gene product, resulting in release of the transcription factor E2F, which increases transcription of the genes required for DNA replication and S phase progression (Fig. 2). Growth arrest is achieved through multiple pathways that inhibit G1 and S phase cyclin kinases. Two families of proteins have been identified that inhibit cell proliferation in G1. One group of proteins comprises the cyclin- or kinase-inhibitory proteins (Cip/Kip) p21, p27, and p57. These proteins share an amino-terminal domain that binds to cyclin D- and E-Cdk complexes and inhibits their activity (29, 68, 87, 117, 159). A second group of proteins comprises the INK4 family, so named because it binds and inhibits Cdk4. This family includes p15, p16, p18, and p19, which exert their effects by binding Cdk4 and displacing the associated cyclin (65). They all contain a fourfold repeated ankyrin-like sequence that bares no homology to the Cip/Kip family. Thus any pathway that impinges on Cip/Kip or INK4 proteins will activate G1 growth arrest.

The tumor suppressor protein p53 is a major regulator of the G1 checkpoint after DNA damage. Cells typically express low levels of p53 that is phosphorylated when DNA damage occurs, resulting in increased stability and its accumulation. In fact, elegant studies showed that p53 accumulates within hours after exposure to IR, ultraviolet (UV) light, or alkylating agents or even after transfection with nicked plasmid DNA or DNase (82, 103). p53 binds to the p21 promoter ~2.4 kb 5′ of the transcription initiation site, resulting in increased levels of p21 mRNA and protein (43, 44). Additional sites with less binding affinity have been identified closer to the TATA box. Thus DNA damage induces p53, resulting in increased levels of p21 that prevent entry into the S phase. A kinetic study (44) with an inducible p53 construct demonstrated that p21 and growth arrest occurred only after p53 levels increased. Interestingly, hydrogen peroxide and other molecules that increase intracellular ROS induce expression of p21 independent of p53 (46, 120). TGF-β- and interleukin-6-type cytokines can also induce p53-independent expression of p21 (12, 38). The growth-inhibitory activities of p21 have been localized to two domains (90). The amino-terminal portion of p21 inhibits cell cycle progression in G1S by binding and inhibiting G1 and S phase kinase activities (127, 159). The carboxy terminus inhibits DNA replication by binding to proliferating cell nuclear antigen (PCNA) and blocking recruitment of DNA polymerase (145). These two domains can act separately from each other to exert growth arrest. As mentioned above, only the amino-terminal domain is found in the related kinase inhibitors p27 and p57 (29, 68, 159).

It is the unique carboxy-terminal PCNA binding domain by which p21 can participate in DNA repair (54). p21 bound to PCNA inhibits binding of DNA endonucleases, such as flap endonuclease (FEN)-1 (35, 135). The PCNA-p21 complex binds to sites of DNA damage, at which point repair is thought to commence when p21 dissociates from PCNA. These biochemical
findings were confirmed at the cellular level with HCT116 colon carcinoma cells in which p21 was deleted through homologous recombination. With these and other cell line models, it was shown that p21-deficient cells are sensitive to DNA damage caused by exposure to cisplatin, nitrogen mustard, or UV radiation (51, 94, 131). Furthermore, p21-deficient cells are unable to repair damaged DNA as shown by their inability to restore reporter activity to a transfected plasmid damaged by the alkylating agent methylmethane sulfonate or UV light. Damage caused by IR appears to be less affected by p21 deficiency; however, this concept was disputed in H1299 pulmonary adenocarcinoma cells containing a regulatable p21 transgene (148). Interestingly, p21-deficient mice show increased sensitivity to hyperoxia as assessed by rapid necrosis of alveolar cells and a 40% reduction in mean survival time (111). Even though hyperoxia failed to inhibit proliferation in these mice, one must consider that the protective effects of p21 may extend beyond simple growth regulation because the mitotic index of the adult lung is very small.

Although TGF-β is not directly regulated by DNA damage or p53, some studies (26, 36) have argued that hyperoxia inhibits proliferation through TGF-β signaling. This scenario is possible because TGF-β increases p53-independent transcription of p21 in keratinocytes (39, 88). TGF-β signals by binding cell surface receptors that lead to activation of intracellular transcription factors called SMADs (see Ref. 71 for a review). The SMAD binding site in the p21 promoter is adjacent to Sp1 sites and is distinct from the p53-binding site (112). The effects of TGF-β to exert G1 growth arrest appear to be cell-type specific because it does not inhibit proliferation only through p21. For example, TGF-β inhibits proliferation of Mv1Lu adenocarcinoma cells by elevating the INK4 protein p15 that displaces p27 from G1 cyclin complexes (125). The increased levels of free p27 can bind tightly to an S phase cyclin A-Cdk2 complex, thereby further preventing phosphorylation of Rb. Although p27 levels are thought to remain relatively constant throughout the cell cycle, they increased in SV40-immortalized type II cells exposed to hyperoxia (36). These findings reveal that hyperoxia activates a G1 checkpoint involving Cip/Kip family members, principally p21. The role of other members of this family, as well as the INK4 family, remains to be examined in oxygen-arrested cells.

S phase checkpoints. There is very little that is known about S phase checkpoints, and it is thought that arrest during this period is catastrophic for cells. In the absence of the G1 checkpoint, damaged cells typically progress through the S phase and arrest in G2. For example, IR induces p21 that inhibits proliferation in G1. Cells lacking p21, such as p21-deficient mouse embryonic fibroblasts or HCT116 cells, progress through the S phase and growth arrest in G2 (41, 146). The cells then continue to replicate their DNA without undergoing cytokinesis and eventually die by apoptosis (147). Thus it is highly intriguing that the same HCT116 cells lacking p21 growth arrest in the S phase when exposed to hyperoxia (122). There are, however, several studies that implicate the existence of S phase checkpoints. For example, low levels of alkylating agents slow S phase progression in Saccharomyces cerevisiae through a mechanism that involves Mec-1, a yeast homolog of the ataxia telangiectasia mutant (ATM), and several other DNA damage checkpoint proteins (113, 114). This decrease in S phase progression did not appear to be dependent on DNA lesions because mec-1-deficient cells rapidly traversed the S phase. Another study (6) demonstrated that nucleotide deficiency could promote S phase arrest that required p53 but not p21. As shown by Neades et al. (102), multiple doses of low levels of UV radiation to mouse keratinocytes induce S phase growth arrest associated with increased Cdk2 kinase activity. This study also showed that p21 was not required for S phase arrest. Interestingly, in another study (14), hyperoxia induced a p21-independent S phase arrest in T47D breast carcinoma cells along with increased Cdk2 kinase activity similar to that in the study by Neades et al. (102). Although there is some evidence that S phase checkpoints may exist and may be activated by hyperoxia, one must also consider that failure to complete the S phase may be due to overall genotoxic stress.

G2 checkpoints. DNA damage also activates a G2 checkpoint through targeting cyclin B and cdc2 kinase activity (Fig. 3). Cyclin B-dependent kinase is the major regulator that moves cells from G2 into mitosis. As cyclin B levels increase in the G2 phase, it binds to cdc2, enters the nucleus, and promotes entry into the M phase. However, this complex remains inactive because of inhibitory phosphorylation on cdc2 at threonine-14 and tyrosine-15. The relative levels of the cdc25 phosphatase and wee1/mik1 kinase maintain this inhibitory phosphorylation. Cells in G2 have higher levels of wee1, whereas cells entering the M phase have higher levels of cdc25. Activated cdc2 kinase phosphorylates cdc25c, resulting in greater phosphatase activity for cdc2 and amplification of cell cycle progression toward mitosis. DNA damage inhibits G2 progression through several pathways. One pathway inhibits activating phosphorylation on cdc25, thereby resulting in decreased cdc25 phosphatase activity (105). Phosphorylated cyclin B-cdc2 complexes are retained in the cytoplasm where they cannot stimulate G2 to M phase progression. A second pathway involves p53-dependent degradation of cyclin B (74). A third pathway involves association of growth arrest and DNA damage (GADD45) with cdc2, leading to disruption of the complex (161). These latter observations were significant because they provided evidence for p53 to inhibit G2 directly by regulating cyclin B expression or indirectly through expression of GADD45 that regulated cdc2 activity.

To date, there is no direct evidence that hyperoxia activates a G2 cell cycle checkpoint (14, 122). G2 growth arrest is also not observed when oxygen-exposed cells are allowed to recover in room air (unpublished observations). There are, however, several suggestive studies that implicate G2 checkpoints. For example, bron-
chial smooth muscle cells exposed to 40% oxygen had more cells in G2 than cultures exposed to 100% oxygen, which accumulated in the G1 and S phases (132). This suggests that cells exposed to lower levels of oxygen were able to complete the S phase and arrest in G2.

Another line of evidence comes from a study that showed that hyperoxia increases p53-independent expression of GADD45 in bronchiolar epithelial and type II cells of adult mice (110). GADD45 was initially identified as an inducible mRNA transcript in CHO cells exposed to a variety of DNA damaging agents (52). It may participate in the G2 checkpoint because it disrupts cyclin B-cdc2 interactions (161). It may also function in DNA repair because antisense expression in cell lines leads to enhanced killing by UV light and cisplatin (136). Because hyperoxia induces GADD45 in type II cells, which are relatively resistant to hyperoxia, one can only speculate at this time about its role in regulating their proliferation.

In summary, there is abundant evidence that hyperoxia alters cell proliferation. Recent studies reveal that part of its growth-arresting properties come from active expression of growth-suppressive molecules including p53, p21, and TGF-β (26, 36, 122, 132). The expression of p21 is probably derived from a response to oxidant DNA damage and expression of p53 (122). We therefore examine evidence for oxygen to damage DNA and how cells sense and activate growth suppression.

HYPEROXIA AND DNA FRAGMENTATION IN THE LUNG

Although the clastogenic effects of hyperoxia have been described extensively in cultured cells like HeLa and CHO cells (34, 56), less is known about its genotoxic effects in the lung. Recent studies (10, 108, 152) in rodent models of hyperoxic lung injury have argued that hyperoxia kills cells by apoptosis because intense terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL)-positive cells, indicative of apoptosis, are detected throughout terminal bronchiolar epithelium and parenchyma. Apoptotic cells undergo orderly DNA fragmentation that may be detected as TUNEL-positive staining and DNA laddering by gel electrophoresis. One paradox with these studies is that DNA laddering has been extremely difficult to detect even though nearly 60% of parenchymal cells exposed to hyperoxia are TUNEL positive. In addition, TUNEL-positive cells are readily detected in bronchiolar epithelial cells that are believed to be more resistant to cell death than parenchymal cells (108). Some studies (10, 150) have attempted to link TUNEL staining with morphological signs of apoptosis and expression of members of the Bcl-2 gene family, which regulate cell survival and death. However, no correlation was found between TUNEL staining, ultrastructural evidence of apoptosis, and expression of the proapoptotic Bax and antiapoptotic Bcl-XL genes (107). Moreover, p53-deficient mice, which failed to induce Bax, showed comparable levels of injury as assessed by TUNEL staining and wet-to-dry lung ratios as in wild-type mice (10, 110). Nevertheless, it is important to recognize that TUNEL staining identifies DNA that contains a break in the phosphodiester backbone of the double helix (8, 62). An alternative hypothesis is that TUNEL staining during hyperoxia reflects DNA damage that either is repaired or will lead to cell death by apoptosis or necrosis. As shown in Fig. 4, TUNEL-positive cells are not easily detected in the lungs of mice exposed to room air. In contrast, faint TUNEL-positive cells are detected throughout the terminal bronchioles and parenchyma after only 12 hours of exposure to a FIO2 > 95%. Because cell death is not observed for an additional 2 days of exposure, one could conclude that TUNEL staining represents DNA strand breaks that need to be repaired or the cells will die.

A second paradox was the findings of Kazzaz et al. (84), who demonstrated that hyperoxia does not induce TUNEL staining in cultured A549 pulmonary adenocarcinoma cells. Similar findings were reported later in the mink lung adenocarcinoma cell line Mv1Lu (123).
In fact, vital dyes revealed that both cell lines died by necrosis. In contrast, hyperoxia induces TUNEL staining in the mouse RAW 264.7 macrophage cell line (115). The single-cell gel electrophoresis or comet assay is a highly sensitive method to discriminate different types of DNA fragmentation caused by genotoxic compounds (13). As shown in Fig. 5, genomic DNA of A549 pulmonary adenocarcinoma cells exposed to room air is intact as shown by the brightly fluorescent nucleus. In contrast, cells exposed to hyperoxia develop a distinct comet tail, consistent with older studies demonstrating the clastogenic nature of hyperoxia (24, 56, 57, 59). Although it remains unclear why TUNEL staining is not detected in most cell lines exposed to hyperoxia, the positive comet and clastogenic effects demonstrating DNA damage are indisputable.

Given that hyperoxia fragments DNA in cells and lungs, one should be able to detect changes in the expression of genes such as the tumor suppressor p53, which increases in cells that have DNA damage (82, 103). In fact, p53 does increase in cell lines like A549 and newborn and adult lungs exposed to hyperoxia (10, 20, 95, 108). These observations were important because p53 regulates transcription of genes that determine whether injured cells cease proliferation and repair DNA damage or undergo apoptosis. Interestingly, one of the first papers to examine the effects of hyperoxia on cell function documented that it altered proliferation of HeLa cells (128). Because molecules that participate in DNA replication are also involved in DNA repair, one can envision that growth arrest promotes repair.

DNA DAMAGE RECOGNITION AND RESPONSES

Although many of the genes that regulate cell cycle progression have been identified, less is known about how cells recognize and activate the DNA damage response. This may be because DNA can be damaged in many different ways, especially within the context of oxidant-induced injury. Studies that will now be presented have revealed that the response to DNA damage is complex and involves genes that recognize DNA damage and transduce the signal to p53, which effects the cellular response by inhibiting proliferation, stimulating repair, or inducing apoptosis (Fig. 6).

Sensors. One of the major unsolved questions in DNA damage signaling today is how cells recognize DNA damage. Initial studies suggested that the DNA-dependent protein kinase (DNA-PK) and poly(ADP-ribose) polymerase (PARP) sensed DNA damage because they bind single-strand DNA. However, genetic evidence has argued that these proteins are not the only players in sensing DNA damage and perhaps are not the major players either. For example, studies have provided evidence for (134, 155) and against (77, 124) the role of DNA-PK to signal p53-dependent growth arrest after IR-induced DNA damage. PARP is a nuclear zinc finger protein that detects single-strand DNA and catalyzes the transfer of ADP-ribose from respiratory coenzyme NAD$^+$ to histones and replication proteins. Poly(ADP-ribosylated) proteins lose their affinity for DNA, which is thought to allow access to DNA. Studies in PARP-deficient mice and cells revealed that PARP participates in base excision repair of DNA that is damaged by IR or alkylating agents such as methylmethane sulfonate (40, 93, 149). As shown in Fig. 4, TUNEL staining in adult mouse lungs exposed to hyperoxia. Adult mice were exposed to room air (A) or 100% fraction of inspired oxygen for 12 h (B), and their lungs were stained for free 3’-hydroxyl ends of DNA as previously described (108). TUNEL-positive nuclei (solid arrows) stained brown, whereas TUNEL-negative nuclei (open arrows) stained blue due to the methyl green counterstain.

Fig. 4. Terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) staining in adult mouse lungs exposed to hyperoxia. Adult mice were exposed to room air (A) or 100% fraction of inspired oxygen for 12 h (B), and their lungs were stained for free 3’-hydroxyl ends of DNA as previously described (108). TUNEL-positive nuclei (solid arrows) stained brown, whereas TUNEL-negative nuclei (open arrows) stained blue due to the methyl green counterstain.

Fig. 5. Single-cell gel electrophoresis (comet) assay. A549 cells were exposed to room air-5% CO$_2$ or 95% O$_2$-5% CO$_2$ (hyperoxia) for 48 h before being embedded in agarose. DNA was separated by electrophoresis, stained with Syber gold, and visualized under fluorescence microscopy. Note the intact nuclear DNA in the room air sample compared with the fragmented DNA that appears like a comet in the sample exposed to hyperoxia.
predicted, PARP deficiency does not alter the sensitivity of cells to UV radiation-induced damage, which is repaired by nucleotide excision repair. Recent studies in Saccharomyces pombe have identified other proteins that are structurally related to PCNA that may act as global DNA damage sensors. The proteins Rad1, Rad9, and Hus1 form a heterotrimer around the DNA helix similar to the homotrimer of PCNA (143). Based on the activities of PCNA, which slides along the DNA as it replicates, one can envision that this complex slides along DNA to identify sites of damage. The observation that Hus1-deficient embryonic fibroblasts are sensitive to damage caused by hydroxyurea or UV light supports the yeast data that these genes may also function as damage sensors in mammalian cells (153). An additional study (100) has suggested that the breast cancer gene BRCA1 can sense double-strand DNA breaks. BRCA1 complexes with a group of proteins, including the mismatch repair enzymes MSH2/6 and MLH2 that are involved in DNA repair and ATM, which transduces the damage response. Collectively, these findings suggest that multiple sensors exist, which complicate studies in ROS-induced damage that produce dozens of different types of damage. For example, PARP may sense hydrogen peroxide-induced damage because inhibitors of PARP can protect myocytes and fibroblasts from hydrogen peroxide-induced death (63). In contrast, hyperoxia does not alter NAD levels, suggesting that PARP, which consumes NAD, may not be a sensor for hyperoxia-induced damage (60).

**Transducers.** Two major groups of proteins have been identified as transducing the signal to downstream regulators such as p53. The first group includes ATM, the ATM-Rad3-related (ATR) gene, and DNA-PK based on their relatedness to the phosphatidylinositol 3-kinase superfamily. Unlike phosphatidylinositol 3-kinase that phosphorylates lipids, these kinases phosphorylate proteins. Because these proteins have weak affinity for DNA, it remains unclear how DNA damage leads to their activation. In response to double-strand DNA breaks such as those caused by IR, ATM phosphorylates p53, murine double mutant (mdm) 2, and Chk2, thereby activating and amplifying additional signals in the DNA damage response (9, 86). It, however, is not the only effector of the damage response because IR still phosphorylates p53 in ATM-deficient cell lines, albeit at a slower rate (25). Interestingly, ATM-deficient cells are highly sensitive to IR-induced damage but not UV radiation or alkylating agents. In contrast, ATR mediates the UV radiation response by phosphorylating p53 (140). It also phosphorylates Chk1 in response to UV radiation, suggesting that DNA damage response may be specified, in part, by their selective activation (89). Thus DNA damage may signal initially through ATM or ATR that can amplify the signal by phosphorylating Chks or by directly activating the DNA sensor p53.

The serine/threonine Chk1 and Chk2 kinases comprise a second group of transducer proteins. These proteins are also phosphorylated by DNA damage and, in turn, phosphorylate p53. For example, ATM phosphorylates Chk2, and it, in turn, can phosphorylate p53 at multiple sites (133). Phosphorylation of serine-20 by Chk1 is thought to be especially important for stabilizing p53 levels in cells. Similar results were observed in another study (89), which showed that Chk1-deficient embryonic stem cells display a G2 cell cycle checkpoint defect in response to IR. IR, UV light, and hydroxyurea also phosphorylated Chk1 through an ATR-dependent mechanism (89). Because nothing is known about the effects of hyperoxia on DNA damage transducers, this remains an open field of study.

**Effectors.** The tumor suppressor protein p53 is the major effector of the DNA damage response because it decides whether injured cells live or die by regulating transcription of the genes involved in cell cycle progression and apoptosis (see Ref. 61 for a review). Although many pulmonary studies focus on p53 mutations associated with lung cancer (reviewed in Ref. 72), their role in lung injury is less studied. The p53 protein may be structurally segregated into three domains. The amino-terminal domain regulates stability and is a transcriptional coactivator, the central domain binds cis-acting elements of gene promoters, and the carboxy terminus regulates transcriptional activity and oligomerization. Under normal conditions, p53 levels are extremely low and the p53 response pathway is considered “off.” DNA damage, hypoxia, nucleotide depletion, telomerase erosion, or uncontrolled growth would be expected to turn on the pathway.
caused by oncogene activation increases p53 expression. p53 levels remain low in cells because it binds the oncoprotein mdm2 that exports it from the nucleus to the cytoplasm where it is ubiquinated and degraded by the proteasome (79, 134) (Fig. 6). mdm2 expression is upregulated by p53, thereby creating a feedback loop by which p53 downregulates its own expression (156). mdm2 is also negatively regulated by p19ARF (p14ARF in the human; p19ARF in the mouse), an alternatively spliced gene encoded within the Cdk p16 gene (118, 163). Thus pathways that alter interactions between p53 and mdm2 or mdm2 with p19ARF regulate p53 expression.

Recent studies suggest that at least three independent pathways regulate p53 levels, of which two pathways lead to site-specific phosphorylation on p53 (81, 103, 126). In fact, a multitude of posttranslational modifications to p53, including phosphorylation of serine-6, -9, -15, -20, -33, -37, and -392, dephosphorylation of serine-376, and acetylation of lysine-320, -373, and -382, have been reported. Although entire review articles have been devoted to this topic (e.g., Ref. 119), only a few key examples are described. The DNA damage transducers involving ATM or Chk2 kinases phosphorylate the amino terminus of p53 at serine-15, consistent with the notion that this blocks association with mdm2. One site by itself is not responsible for stabilizing p53 because IR still induced expression of a mutant form of p53 where serine-15 was converted to alanine (25). However, this mutant failed to elicit p53-dependent apoptosis. Recently, phosphorylation on serine-20 was shown to stabilize p53 through Chk1 and independent of ATM or ATR (28, 133). A second pathway involves ATR-dependent activation of p53 by UV light and chemotherapeutic drugs. ATR phosphorylates serine-15, -37, and -392 (392 in humans; 389 in mice) in response to UV radiation (67, 81). Interestingly, ATR phosphorylates serine-15 and -392, whereas ATM phosphorylates serine-15 but not serine-392. Thus the UV radiation response has overlapping and distinct effects on p53 phosphorylation. Oncogene-dependent cell growth represents a third pathway to regulate p53 as shown by a recent study demonstrating that activation of Ras induced the Raf/mitogen-activating protein kinase mitogen-activating protein kinase pathway, resulting in increased mdm2 transcription and p53 degradation (126). Although hypoxia increases p53 in vivo and in vitro, little is known about how this occurs. A recent study (122) showed that hypoxia activates p53-dependent transcription and phosphorylates p53 on serine-15, consistent with a DNA damage response involving ATM and/or ATR kinases. Additional studies that examine whether hypoxia phosphorylates other sites on p53 are needed to provide insight into signal transduction pathways activated by oxygen-induced DNA damage.

In addition to regulating p53 abundance, additional changes must occur before it can mediate transcriptional activity. A major unresolved question in the field is how cell growth and apoptosis are discriminated by p53. Vousden (144) has recently proposed two models.
Given that hyperoxia inhibits proliferation through activation of cell cycle checkpoints, how does this occur and does it really protect cells from hyperoxia? This question may seem strange when one considers that the mitotic index of the endothelial cell is extremely small and the type I cell is terminally senescent. Similarly, if growth arrest is beneficial, why do newborn lungs resume proliferation during continuous exposure without fixation of mutations in their genome? Once repair has been completed, what are the molecular signals that stimulate reentry into the cell cycle and how does the repaired lung terminate proliferation so as to not become fibrotic? Another question is whether hyperoxia-induced cell injury can prevent or promote cell injury caused by another agent. The rationale for this thought is that both hyperoxia and carcinogens target p53. Interestingly, studies have shown that hyperoxia or ozone can modify the pulmonary response to carcinogens (see Ref. 154 for a review). Answers to these and other related questions may provide novel insights into the cellular response to oxidant injury caused by hyperoxia as well as by IR, bleomycin, ozone, nitrogen dioxide, particles, inflammation, and ischemia-reperfusion. Given the relevance of oxygen toxicity to produce ROS, the role of which in signal transduction processes has recently been appreciated by the entire scientific community, I anticipate that studies on the cellular response to hyperoxia will be quite prolific in the future!

Because no review can cover the myriad of studies related to one topic, I apologize for those that were not included due to space constraints. I am extremely grateful to the members of my laboratory, especially Rhonda Staversky, and my colleagues who have contributed to the research presented in this review article.

This work was supported in part by National Heart, Lung and Blood Institute Grant HL-58774.

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