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Forum Review

Pulmonary Antioxidant Defenses in the Preterm Newborn with Respiratory Distress and Bronchopulmonary Dysplasia in Evolution: Implications for Antioxidant Therapy

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ABSTRACT

Preterm neonates with respiratory distress are exposed not only to the relative hyperoxia *ex utero*, but also to life-saving mechanical ventilation with high inspired oxygen (O_2) concentrations, which is considered a major risk factor for the development of bronchopulmonary dysplasia, also referred to as chronic lung disease of infancy. O_2 toxicity is mediated through reactive oxygen species (ROS). ROS are constantly generated as by-products of normal cellular metabolism, but their production is increased in various pathological states, and also upon exposure to exogenous oxidants, such as hyperoxia. Antioxidants, either enzymatic or nonenzymatic, protect the lung against the deleterious effects of ROS. Expression of various pulmonary antioxidants is developmentally regulated in many species so that the expression is increased toward term gestation, as if in anticipation of birth into an O_2-rich extrauterine environment. Therefore, the lungs of prematurely born infants may be ill-adapted for protection against ROS. While premature birth interrupts normal lung development, the clinical condition necessitating the administration of high inhaled O_2 concentrations may lead to permanent impairment of alveolar development. An understanding of the processes involved in lung growth, especially in alveolarization and vascularization, as well as in repair of injured lung tissue, may facilitate development of strategies to enhance these processes. *Antioxid. Redox Signal.* 6, 155–167.

OVERVIEW

OXYGEN (O_2) TOXICITY is mediated through reactive oxygen species (ROS), *e.g.,* superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2), which are generated endogenously by several mechanisms under both physiological and pathological conditions (46, 71). The major source of ROS in the lung is the mitochondrial respiratory chain, in which the four-step reduction of molecular O_2 to water is coupled to the vital production of cellular ATP, *i.e.,* oxidative phosphorylation. Under physiological conditions, not more than ~1–2% of the O_2 entering the respiratory chain exits as $O_2^{\cdot -}$. Under hyperoxic conditions, however, the pulmonary mitochondrial production of $O_2^{\cdot -}$ is enhanced in linear relation to O_2 tension (45). ROS have important roles in cell signaling and protection against microorganisms, but are potentially harmful because of their damaging effects on proteins, lipids, and DNA, *i.e.,* virtually all components of the cell (107). Furthermore, one source of ROS production can set off additional sources, thereby amplifying the initial damage.

To protect the host against deleterious effects of ROS, an elaborate antioxidant system, including both enzymatic and nonenzymatic agents, has evolved (Fig. 1). Traditionally, antioxidants have been defined as substances that (a) prevent the formation of ROS or other oxidants, (b) scavenge them, or (c) repair the damage that they cause. The complex endogenous antioxidant system consists of, for example, classical antioxidant enzymes (AOEs), glutathione (GSH), and thiore-

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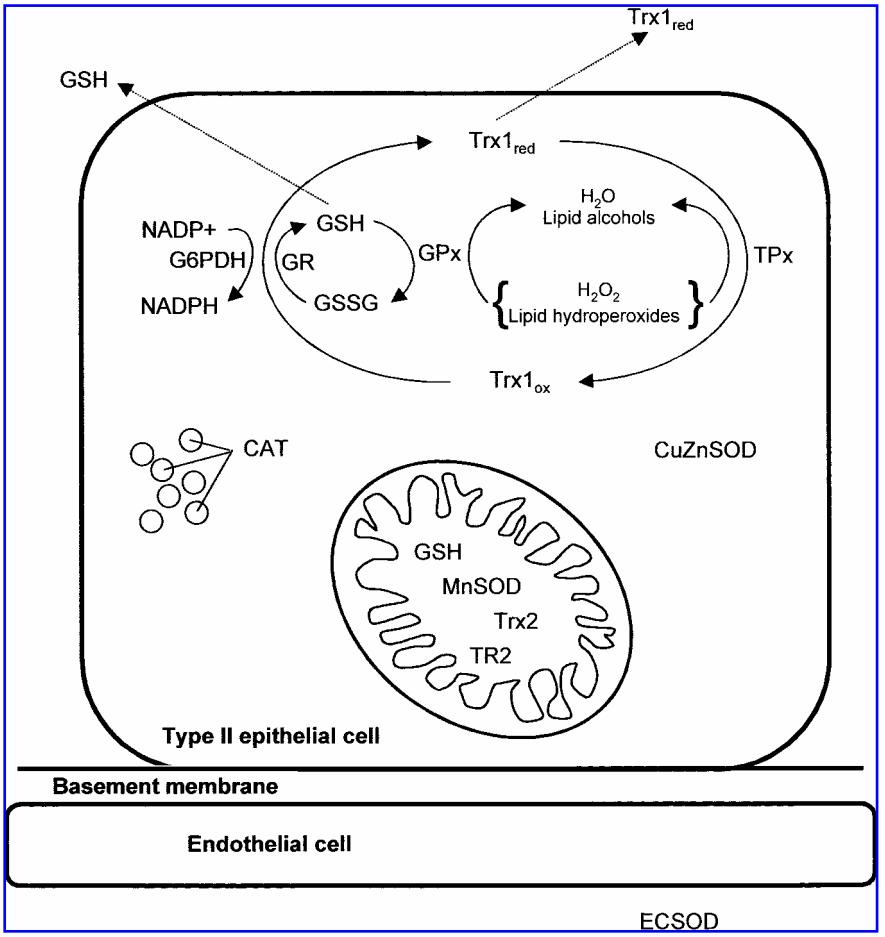

FIG. 1. A schematic diagram of antioxidant defenses in lung epithelial and endothelial cells. CAT, catalase; CuZnSOD, copper-zinc superoxide dismutase; ECSOD, extracellular superoxide dismutase; G6PDH, glucose 6-phosphate dehydrogenase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; MnSOD, manganese superoxide dismutase; NADP, nicotinamide adenine dinucleotide phosphate; TPx, thioredoxin peroxidases (also called peroxiredoxins); TR, thioredoxin reductase; Trx, thioredoxin; Trx_{ox}, oxidized thioredoxin; Trx_{red}, reduced thioredoxin.

doxin (Trx) with their associated redox cycles, heme oxygenases, and numerous small-molecular-weight antioxidants, including vitamins C and E. Potential for ROS-mediated cell injury exists if the production of ROS exceeds the antioxidant capacity of the cell.

In animal models, the expression of the most important pulmonary AOEs increases toward term gestation (40, 97, 114). Thus, it can be hypothesized that, owing to the immaturity of antioxidant defense systems, the lung of the prematurely born may be exceptionally vulnerable to O_2-induced injury unless capable of mounting an adequate antioxidative response. In numerous experimental models, tolerance to hyperoxia has been ascribed to increased expression of AOEs, especially to that of the mitochondrial manganese superoxide dismutase (MnSOD) (18, 109, 123). It has also become clear from animal studies that the developmental regulation of AOEs varies between species, especially regulation in response to hyperoxia (40). Given these interspecies variations, the results obtained from studies on lower species cannot be directly extrapolated to human development. Our laboratories have therefore focused on elucidating the expression and regulation of AOEs during ontogenesis and in hyperoxia in a nonhuman primate model of bronchopulmonary dysplasia (BPD), as well as in human preterm neonates. In the baboon model of BPD, preterm animals breathing 100% O_2 develop chronic lung disease within 7–14 days. The pathologic, morphometric, and biochemical patterns represent those seen in human neonates with mild to moderate clinical BPD who survive. The baboon model is, by far, the best characterized experimental model of chronic lung disease associated with premature birth (21).

OXYGEN-INDUCED LUNG INJURY

The respiratory tract is the primary target for O_2-induced injury, because it, in contrast to other tissues, comes into direct contact with inhaled O_2 and other exogenous oxidants. The degree of O_2-induced damage depends on O_2 concentration and length of exposure. Oxygen concentrations of >95%

for >3 days are lethal for adult animals of many lesser species (40), which die with signs of progressive respiratory distress (26). The primary injury is to the lung, where typical morphological changes can be detected in a defined temporal sequence. The initiation phase (~24–72 h of exposure) is characterized by increased production of ROS, but no structural changes are evident yet. The next step is the rapid accumulation of inflammatory cells into the intravascular and interstitial spaces, followed by release of inflammatory mediators. The appearance of neutrophils is associated with amplification of the extent of lung injury. The earliest structural changes are detected in capillary endothelium, which, when damaged, will allow increased capillary permeability and pericapillary edema. In the lungs of adult rats exposed to 100% O_2, ~50% of capillary endothelial cells are destroyed prior to death (26). Exposure of rats to lethal concentrations of O_2 is associated also with morphological changes in type I and II pneumocytes, but no alveolar epithelial cell loss has been documented (25). In primates, however, there is a progressive loss of type I cells and a reactive hyperplasia of type II cells, which are progenitor cells of the alveolar epithelium (69). Very similar changes have been identified in lungs of humans exposed to hyperoxia (81).

Most species can tolerate sublethal concentrations of O_2, *i.e.,* 50–85% O_2, for several weeks (23, 58). Although animals exposed to sublethal, or adaptive, hyperoxia survive, they nevertheless develop a lung injury, the manifestation of which is largely similar to that in lethal hyperoxia, but the onset and progression are delayed. For example, ~40% of capillary endothelial cells are destroyed by the seventh day of exposure of rats to 85% O_2, after which no further destruction occurs. The interstitial inflammatory cells in adaptive hyperoxia are monocytes rather than neutrophils. Furthermore, the lung injury caused by exposure to sublethal O_2 concentrations is characterized by increased proliferation of type II pneumocytes and fibroblasts, and by increased deposition of collagen into the interstitium, leading to interstitial fibrosis and development of secondary pulmonary hypertension (25, 26). Enlargement of terminal air spaces and destruction of alveolar septal walls have also been observed. The adaptive changes described have been characterized in both rats and primates, including humans (25, 69).

PULMONARY ANTIOXIDANT DEFENSES

Superoxide dismutases (SODs)

Eukaryotes have three different SODs: mitochondrial MnSOD, cytosolic copper-zinc SOD (CuZnSOD), and extracellular copper-zinc SOD (ECSOD) (48, 82). Of the total intracellular SOD activity, MnSOD constitutes ~10–15% and CuZnSOD 85–90%. ECSOD is the major SOD in the extracellular matrix and fluids. Dismutation of two $O_2^{\cdot-}$ radicals can also occur spontaneously, but the reaction catalyzed by SOD is 10^4 times faster at neutral pH (46). As discussed above, O_2 tolerance has been ascribed to MnSOD in numerous experimental models, and therefore its role and regulation are discussed in more detail. The essential role of MnSOD has been further demonstrated by a gene knockout mouse model (78).

MnSOD (product of the *SOD2* gene) is a manganese-containing, homotetrameric enzyme with a molecular mass of 88 kDa. MnSOD is located in the mitochondrial matrix and is considered to be the primary defense against $O_2^{\cdot-}$ released from the mitochondrial electron transport chain (48). *SOD2*, on chromosome 6, is a single copy gene consisting of five exons interrupted by four introns. It is transcribed as two major mRNA species of 4 kb and 1 kb in size (116). These transcripts have identical coding regions but differ in the length of their 3′-untranslated region, and the turnover rate of the 4-kb transcript is faster than that of the 1-kb transcript (85). MnSOD is translated as a precursor protein with a 24-amino acid mitochondrial leader peptide, and is imported posttranslationally into the mitochondria (127).

MnSOD is induced by several factors both *in vivo* and *in vitro*. These include, for example, hyperoxia (47), H_2O_2 (118), reductants, such as dithiothreitol, β-mercaptoethanol, *N*-acetylcysteine (NAC), and Trx (27, 28), inflammatory cytokines, such as tumor necrosis factor-α, interleukin 1, and interferon-γ (113, 128), lipopolysaccharide (18), peroxynitrite (64), cigarette smoke (52), and fibrogenic material including asbestos fibers (66). Furthermore, exposure to a combination of various cytokines, or cytokines and lipopolysaccharide, leads to a synergistic induction of MnSOD (113). Moreover, the regulation of MnSOD in response to oxidants is complex and has been reported to occur at both pre- and posttranslational levels (20). In hyperoxia, the time course or magnitude of MnSOD induction at the mRNA level is not always paralleled by increases in immunoreactive protein or enzyme activity (12, 18, 57). The MnSOD promoter contains potential binding sites for several redox-sensitive transcription factors, including activator proteins 1 and 2 (AP-1 and AP-2), promoter-selective transcription factor 1 (SP-1), and nuclear factor κB (NF-κB) (116). In some studies, induction of MnSOD following oxidant exposure has been associated with NF-κB and AP-1 activation (27). It should be noted that, although there is substantial experimental evidence to link the induction of AOEs, especially that of MnSOD, to development of tolerance to hyperoxia, contradictory results have also been obtained (6, 95).

H_2O_2-decomposing enzymes

Catalase (CAT) and glutathione peroxidase (GPx) are complementary mechanisms in metabolizing H_2O_2 so that CAT scavenges H_2O_2 efficiently at high H_2O_2 concentrations and GPx, in turn, at low H_2O_2 concentrations. GPx is part of the GSH redox cycle, in which GPx utilizes reduced GSH as a cofactor to metabolize H_2O_2 and the resulting oxidized GSSG is then recycled back to GSH at the expense of NADPH generated by glucose 6-phosphate dehydrogenase. In addition to serving as a cosubstrate for GPx, GSH has also several other important functions, including direct radical scavenging, conjugation reactions, and maintenance of reduced thiol status of proteins (11).

Trxs are low-molecular-weight redox-active proteins that are important in cellular proliferation, signal transduction, and antioxidant function. Furthermore, Trx is a potent protein

disulfide oxidoreductase that also functions as cosubstrate for thioredoxin peroxidases (TPx), also called peroxiredoxins. In analogy with the GSH redox cycle, oxidized Trx is converted back to reduced form by the NADPH-dependent flavoprotein thioredoxin reductase (TR) (59). In addition, another recently described Trx-dependent peroxidase, *l*-Cys peroxiredoxin, also may have an important role in limiting membrane damage and assuring cell survival in *in vitro* models (80, 94).

Other antioxidants

Antioxidant defenses also include a number of other small-molecular-weight and enzymatic antioxidants. For example, α-tocopherol (vitamin E) is a lipophilic radical scavenger present in biological lipid phases, where it functions to prevent lipid peroxidation. The resulting tocopherol radical is recycled back to α-tocopherol by water-soluble ascorbate (vitamin C). Moreover, binding of transition metals and toxic heavy metals is an important part of antioxidant defense. For example, metallothioneins are cysteine-rich metal-binding proteins that have cytoprotective effects against oxidative stress (75, 96, 100). Other metal-chelating proteins include, for example, transferrin, ferritin, lactoferrin, ceruloplasmin, and albumin (54). Heme oxygenases 1 and 2 (HO-1 and HO-2, respectively) are enzymes whose activity leads to accumulation of the antioxidant bilirubin and the potentially cytoprotective signaling molecule carbon monoxide, and degradation of the prooxidant heme. Overexpression of HO-1 has been shown to protect against hyperoxia (91, 106). Surprisingly, gene inactivation of HO-1 in mice results in protection against hyperoxia, possibly through decreased levels of free iron (35, 98). On the other hand, HO-2 knockout mice have elevated levels of redox-active iron, and are sensitized to hyperoxia (34). Other antioxidative compounds include, for example, ubiquinol, β-carotene, uric acid, and flavonoids.

PULMONARY ANTIOXIDANT DEFENSES DURING ONTOGENESIS

Five stages have been identified in human fetal and postnatal lung development. The ability for pulmonary respiration is one of the main determinants for survival of any preterm neonate. This usually occurs in the canalicular period after 24 gestational weeks, when the blood–gas barrier has been formed and surfactant synthesis begins. Following birth into an O_2-rich extrauterine environment (21% O_2), the lung cells are exposed to several fold higher O_2 concentrations than *in utero* (3% O_2). Moreover, several clinical conditions of the newborn necessitate the administration of ventilatory support, sometimes with as high as 100% O_2 concentrations. Some lung cell subpopulations, such as bronchial and alveolar epithelial cells, are directly exposed to high inhaled O_2 concentrations due to their anatomical locations.

The maturation of the surfactant system during the final one-third of term gestation is paralleled by increased expression of pulmonary AOEs in various mammals (97, 114). The late-gestational increase in AOE activities has been considered to occur in preparation for birth into an O_2-rich environment. Beginning of air breathing induces some pulmonary antioxidants, such as ECSOD (89) as shown by enhanced activity levels shortly after birth. On the other hand, exposure of 8-week-old mice to very high levels of O_2 may cause a reduction in cell ECSOD, an effect that may be related to the developmental stage (92). Furthermore, ECSOD is localized intracellularly in preterm and term rabbit lungs, and secretion of active ECSOD into the extracellular compartment increases with age (89). The transition from fetal O_2 tensions to air or O_2 breathing at birth also affects the Trx redox cycle. We have previously shown that Trx, TPx-I, and TR are expressed constitutively at low levels during baboon lung development. However, the mRNA levels for Trx, TPx, and TR were significantly increased after air or O_2 breathing in both preterm (125 or 140 days corresponding to 24 and 27 gestational weeks, respectively, in human development) and term (186 days) baboons (29, 30). TPx-I, but not TPx-II, has been shown to increase during late gestation and in response to air or O_2 breathing in rat lung (70).

The level at which AOEs are regulated in late gestation varies between enzymes (17). Furthermore, redox-sensitive mRNA-binding proteins for MnSOD and CAT have been characterized (16, 39), the former being developmentally regulated so that there is less MnSOD mRNA-binding activity in adult rat lung as compared with prenatal or neonatal lung (39).

Our studies on expression and regulation of AOEs in human lung during development showed that the mRNAs for pulmonary MnSOD, CuZnSOD, and CAT were increased toward term and adulthood (4). However, in terms of activity, only CAT increased in correlation with the respective mRNA, whereas the activities of the other AOEs were unchanged (4). Our results on pulmonary CAT activity confirm those obtained previously (84) and extend them by showing that the increase in activity is due, at least in part, to increases in steady-state mRNA levels.

The developmental regulation of MnSOD is the subject of some controversy. We found that the increase in MnSOD mRNA is not paralleled by increases in enzyme activity or immunoreactive protein in human lung during development (4, 5). Our results on MnSOD activity and protein are in keeping with those obtained by Strange *et al.* (104), but are in disagreement with another study (36), in which some developmental increase in MnSOD protein was detected in peripheral airways. The latter study is supported by observations from baboon studies, where lung MnSOD specific activity increases during the final third of fetal life (87). We found the localization of MnSOD to be widespread to various cell types during human lung ontogenesis. As previously reported for rat lung (24, 88), the most intense staining was found in bronchial and alveolar epithelium (5, 36). These cell types directly encounter inhaled O_2, and it is therefore logical that they should have a high basal level of MnSOD.

Pulmonary GPx mRNA was unchanged during development and, in agreement with animal studies (32, 119), GPx activity declined postnatally. Two previous studies on GPx in human lung reported large interindividual variation, but no developmental trend was observed (49, 84). A previous study from our laboratory showed that the activity of pulmonary glutamate-cysteine ligase (previously known as γ-glutamylcysteine synthetase) also is similar in fetuses as in term neonates and adults (77). However, it is known that the concentrations of GSH in plasma and bronchoalveolar lavage fluid in preterm human infants is lower than in term infants (65).

Therefore, even though the GSH synthetic capacity in several tissues of preterm human neonates appears similar to that of term neonates (77), GSH synthesis in these infants may be impaired due to lack of substrate (112). Trx has been detected in epithelial structures of human fetal lung during the second trimester (50), but the developmental regulation of Trx in human lung is not known.

PULMONARY ANTIOXIDANT DEFENSES IN RESPIRATORY DISTRESS SYNDROME (RDS) AND BPD

The role of O_2 toxicity in the pathogenesis of BPD has been extensively studied. These studies clearly demonstrate that the histological findings in experimental pulmonary O_2 toxicity are strikingly similar to those seen in BPD (25, 81). Typical histopathological findings include endothelial and epithelial cell damage, bronchial smooth muscle hypertrophy, interstitial fibrosis, and simplification of the acinar structure with reduction in total number and surface area of alveoli. The involvement of ROS in the pathogenesis of BPD is further supported by indirect evidence from several human studies (99). The pathogenesis of BPD is now known to be multifactorial and, apart from O_2 therapy, other predisposing factors include prematurity, high positive airway pressures, inflammation, pneumonia, meconium aspiration, lung hypoplasia, undernutrition, and genetic predisposition (68).

As discussed above, an increase in pulmonary AOE activities is mandatory for survival under hyperoxic conditions in many animal species. The regulation of pulmonary AOEs in hyperoxia is complex, and differs between animal species and also between young and old individuals of the same species (40, 129). For example, term neonates of mice, rats, and rabbits, but not of guinea pigs and hamsters, can tolerate higher concentrations of O_2 for longer periods of time than adults of the same species (43). Oxygen tolerance in the surviving neonates is associated with rapid increases in lung AOE expression (40, 129). In response to early neonatal hyperoxia, the regulation of AOEs is uniform in rat lung, *i.e.,* increase in mRNA concentration occurs through prolongation of mRNA half-life (17). In contrast to term neonates, AOE regulation, at least that of CuZnSOD, in adult rats exposed to hyperoxia is translationally controlled (55). The differences in AOE regulation may explain in part why neonatal animals of many species are more tolerant of hyperoxia than mature animals of the same species. Furthermore, adaptation of the newborn lung to hyperoxia has been associated with increased expression of surfactant proteins (125).

Due to the developmental regulation of AOEs, preterm babies with immature antioxidant defense systems may be exceptionally vulnerable to O_2-induced lung injury, unless they are capable of rapidly mounting an antioxidative response when exposed to hyperoxia. Indeed, preterm baboons and rabbits are unable to increase the activities of MnSOD, CuZnSOD, CAT, GPx, and glucose 6-phosphate dehydrogenase when challenged with hyperoxia, and consequently are more susceptible to O_2-induced injury (42, 87). The mRNA levels for MnSOD and CuZnSOD, however, were increased in the preterm baboon (125 and 140 days corresponding to 69% and 78% of term pregnancy, respectively) in response to O_2 treatment, suggesting that the failure to increase respective enzyme activity levels occurs at the posttranscriptional level (87). However, the 140-day preterm baboon is capable of increasing MnSOD protein in response to bacterial infection. This increase in MnSOD protein concentration occurs despite decreases in MnSOD mRNA, again suggesting posttranscriptional regulation (19). Further illustrating the differences in perinatal antioxidant regulation between species, preterm guinea pigs and rats show hyperoxia-induced increases in AOE activities resulting in relative tolerance to hyperoxia (15, 102).

The regulation of the Trx redox cycle does not appear developmentally regulated toward term, but instead its expression appears to be controlled primarily by ambient O_2 tensions postnatally (29, 30). We have previously shown that 140-day preterm baboons breathing 100% O_2 have greater Trx, TPx, and TR mRNAs after 1, 6, or 10 days of life than fetal control animals. In fetal distal lung explant culture, mRNAs for Trx, TPx, and TR were elevated within 4 h in 95% O_2 relative to 1% O_2, and the response was similar at various gestations. In contrast, Trx activity did not increase in lung explants from preterm animals (125 or 140 days), but did in those from near-term (175-day) fetal baboons after exposure to hyperoxia. Preterm baboons of 140 days of age showed increased TPx activity when exposed to as-needed O_2, but decreased activities when exposed to 100% O_2. When Trx redox status was determined, increased O_2 tension shifted Trx to its oxidized form. The regulation of Trx, TPx, and TR under hyperoxic conditions in this model appears transcriptional, because treatment of lung explants with actinomycin D inhibited the increase in mRNA levels (29, 30).

Expression of MnSOD in human patients with RDS and BPD has been investigated by several research groups (36, 104), including our own (5). We found that the amount of immunoreactive MnSOD protein in preterm patients (24–29 gestational weeks) with RDS who had been treated with $\geq$70% inhaled O_2 concentrations for a minimum of 32 h was not increased in any of the pulmonary cell subpopulations studied. There was one exception, however, because the staining pattern for MnSOD in arterial endothelium was more intense in RDS patients than in age-matched controls. As the partial pressure of O_2 in the pulmonary artery is low, the increased MnSOD in arterial endothelium could not represent an adaptive response to hyperoxia. We speculated that this finding could be accounted for by laminar shear stress as a result of surfactant treatment, lung volume recruitment, and the subsequent increase in blood flow in pulmonary artery. Laminar shear stress has been shown to induce MnSOD (108) and several other antioxidants in experimental models (14).

We detected no changes in MnSOD expression in BPD patients (5). This was somewhat surprising, because it is very likely that BPD patients had experienced pulmonary inflammation resulting from long-term O_2 treatment and infections. It is possible, however, that MnSOD was up-regulated in the lungs of BPD patients during the acute phase of the inflammatory reaction, and had returned to baseline levels by the time the patients died of BPD at the mean age of 3.5 months. Our results on MnSOD expression in RDS and BPD are compatible with results from a previous study (104), but contra-

dict those of another (36). In the latter study, MnSOD expression was slightly decreased in type II pneumocytes of two RDS patients, and moderately increased in type II cells of one BPD patient, in whom considerable type II cell proliferation had occurred. Based on the present results, it can be concluded that preterm human neonates have a limited ability to induce MnSOD in response to O_2 treatment, and this may be reflected in the poor outcome. It has to be emphasized, however, that the RDS and BPD patients in this study died from their illnesses, and therefore, the results may not reflect events occurring in infants who recover.

THERAPEUTIC IMPLICATIONS

Substantial advances into understanding the pathophysiology and pathogenesis of RDS and BPD have been made, and these have been reflected in various therapeutic interventions. These include, for example, exogenous surfactant to reduce the respiratory distress, and antenatal glucocorticoids (67). In animal models, the beneficial effects of antenatal glucocorticoids may arise not only from the maturational effects on surfactant production, but also from acceleration of maturation of AOEs (44). Natural lung surfactant also has both direct (inhibition of lipid peroxidation, SOD and CAT activity) and indirect (antiinflammatory actions) antioxidant effects (9, 83, 130).

In spite of the beneficial effects of prenatal corticosteroids and exogenous surfactant replacement therapy on survival, especially when combined with modern, less traumatic, ventilatory techniques, high morbidity still persists in very preterm newborns with RDS (38). Use of room air in resuscitation of preterm babies has been evaluated in controlled studies (111), and 100% O_2 is not automatically used in resuscitation of these babies, unless required. Treatment with inhaled nitric oxide ($^{\cdot}NO$) has been found to improve oxygenation, especially in populations with pulmonary hypertension, thereby reducing the need for very high, inhaled O_2 concentrations. NO therapy also can decrease lung neutrophil accumulation in preterm human neonates with hypoxemic respiratory failure (72). By and large, theoretical toxicities of $^{\cdot}NO$, often described in experimental settings where very high $^{\cdot}NO$ concentrations (80–100 ppm) have been used, have not been borne out in clinical experience with newborns treated with much lower (5–6 ppm) $^{\cdot}NO$ concentrations (3).

The rationale for antioxidant therapy is based on the belief that the individual is deficient in endogenous antioxidant defenses due to developmental regulation and/or as a consequence of overwhelming oxidative stress because of the clinical condition *per se* and/or its treatment. Upon recognition of ROS in the pathogenesis of BPD, a wide variety of antioxidants and means of delivery have been studied in order to prevent or ameliorate the severity of BPD. Some of these approaches have been more beneficial than the others. Common problems encountered in *in vivo* studies are, for example, poor cell penetrance, short plasma half-life, and antigenicity. Many of the natural antioxidants have a specific, intracellular, often organellar, site of action, and therefore, delivery or expression of antioxidants at their natural site of expression is extremely challenging. To overcome some of these problems, new synthetic compounds, such as catalytic antioxidants with SOD and/or peroxidase activity, have been tested in preventing ROS-induced injury. Recent investigation showed for the first time that one of the catalytic antioxidants, a metalloporphyrin AEOL 10113, may have beneficial effects in prevention of BPD in preterm baboons (13).

Some studies indicate a favorable role for several antioxidant compounds in the treatment of lung injury. For example, intravenous or intraperitoneal administration of liposome-encapsulated or polyethylene glycol-conjugated CuZnSOD and CAT provides protection against O_2 (124). Intratracheal administration of AOEs, such as MnSOD, CuZnSOD, and CAT, has been evaluated in ROS-mediated injury, with promising results in various animal species (93, 115, 121).

Augmentation of GSH-dependent antioxidant defense has been extensively studied, with the rationale that GSH synthesis may be limited in the preterm neonate (65, 112). Intravenous GSH has been shown to have clinically and biochemically beneficial effects in the preterm baboon, as indicated by normalization of circulating cysteine and GSH, but, nevertheless, the alveolar-arterial O_2 gradient was increased, indicating worsening oxygenation (103). Some studies have elucidated the role of cysteine precursors, *e.g.,* NAC, in prevention of lung injury. NAC has been found to improve oxygenation and shorten the duration of acute lung injury in adult patients (8, 105). Other studies, however, have been unable to confirm any beneficial effect on oxygenation, requirement for ventilatory support, or survival (37).

Based on promising data from *in vitro* and *in vivo* studies in animals, some of the antioxidants have reached human trials aimed at preventing BPD. Intramuscular high-dose vitamin A administered over a four-week period slightly decreased the risk of BPD in extremely-low-birth-weight infants (110). Vitamin E, on the other hand, failed to show any positive effects in preventing BPD (120). Prenatal administration of thyrotropin releasing hormone to stimulate AOEs and surfactant maturation seemed promising, but in fact was associated with severe adverse effects in a clinical trial (7). Treatment of human preterm babies with intratracheal recombinant CuZnSOD (2.5 mg/kg or 5 mg/kg rhCuZnSOD every 48 h, up to seven doses, in a placebo-controlled and randomized study) increases activity of this enzyme in intratracheal fluid, serum, and urine and reduces markers of acute lung injury (neutrophil chemotactic activity, albumin concentration) in tracheal aspirate but, according to preliminary results, does not affect the primary outcomes of death or BPD at the mean age of 28 months corrected age (31). However, administration of CuZnSOD was associated with less severe intraventricular hemorrhage and periventricular leukomalacia. In a Nordic double-blind multicenter trial, a 6-day course of intravenous NAC (16–32 mg/kg/day) did not prevent BPD or death in extremely-low-birth-weight infants (2).

The possibility of gene therapy, defined as introduction of new genetic material into cells of an individual with resulting therapeutic benefit, in the prevention and treatment of ROS-mediated cytotoxicity is under intense investigation. Human gene therapy was originally conceived as an approach to treat hereditary genetic diseases, such as cystic fibrosis or α_1-antitrypsin deficiency, but could be applied to acquired or developmental conditions as well (122). In theory, the advan-

tage of gene therapy lies in the possibility that the desired long-lasting therapeutic effect is achieved by single or repeated local administration of a gene. However, several limitations need to be resolved before pre- or postnatal gene therapy of human neonates to prevent or alleviate O_2-induced lung injury becomes a reality. These include, for example, identification of the candidate genes, which would be either over- or under-expressed, issues related to vector optimization, and potent, adverse inflammatory and/or immune responses of the host.

Although transcription and growth factors are not understood as "traditional" antioxidants, their action can lead to prevention or repair of ROS-induced damage, and therefore, they could fulfill the definition of an antioxidant. Results from experimental studies utilizing these novel approaches suggest that modulation of certain transcription factors and signaling pathways, whose activation results in regulation of a variety of antioxidant defenses, might present a powerful way to affect outcomes in oxidative stress. Likewise, accumulating experimental data suggest that enhancing DNA repair mechanisms, such as apurinic/apyrimidinic endonuclease 1 (APE-1) or APE-1/Ref-1 (56), promoting growth arrest (76, 90), and paradoxically, inducing growth factors (117) may have beneficial effects in the prevention and repair of lung injury.

DISCUSSION

Oxygen remains a necessary and often life-saving therapy in the treatment of critically ill neonates. Attributable to advances in neonatal intensive care, an increasing proportion of very-low-birth-weight preterm babies survive today. However, the risk of developing BPD increases with decreasing birth weight and gestational age. An understanding of the molecular mechanisms whereby protection against pulmonary O_2 toxicity can occur may enable development of targeted and effective interventions aimed at preventing or alleviating neonatal O_2-induced lung disease. ROS are not, however, merely harmful, because they also have functional roles in, for example, killing of microorganisms and modulation of signal transduction cascades culminating in, for example, regulation of cell growth, proliferation, and differentiation (107). Antioxidant treatment of neonatal lung disorders merits special attention because ROS may exert pivotal functional roles in lung growth. The goal of optimal antioxidant therapy is to scavenge the excess of harmful radicals without interfering with essential cellular functions.

As discussed above, the developmental profile of pulmonary AOEs in lesser species has been well characterized, and the results show that AOE regulation varies between species during ontogenesis and especially in hyperoxia. It follows that results obtained from studies on lesser species cannot be directly extrapolated to primates, including humans. A warning example comes from proof-of-principle studies in developing rats where lung growth-suppressive effects of hyperoxia could be overcome to a considerable effect by administration of the iron chelator deferoxamine (41). However, when given to the preterm, O_2-breathing, baboon in an attempt to inhibit iron-catalyzed free radical generation and lessen the severity of O_2-induced pulmonary injury, deferoxamine was rapidly fatal, with the majority of animals developing a fulminant cardiovascular collapse (33). Such studies demonstrate the extreme value of the *preterm* primate model. In studies from our own laboratory, we have learned that deferoxamine can decrease markedly the reactivation cycle of iron-sulfur cluster-containing enzymes, such as aconitase (51). Furthermore, subsequent studies have shown that very preterm baboons are especially susceptible to aconitase inactivation under hyperoxic conditions (86). Thus, deferoxamine may have been helpful in preventing lung hypoplasia in the term neonatal rat in hyperoxia (41), where compensation for abnormal energy metabolism may have occurred through induction of glycolytic enzymes, but such compensation was not possible in the case of the preterm primate.

From our studies in preterm baboon (29, 30, 87) and human (4, 5) infants, it can be concluded that there is no coordinated up-regulation of AOE activities during ontogenesis. Therefore, in terms of pulmonary AOE capacity, preterm primate neonates *under physiological conditions* appear not to be at a disadvantage as compared with term neonates. However, the ability to induce AOE activities *in response to hyperoxia* may be differentially controlled in preterm and term primates. Results from our studies indicate that preterm human and baboon neonates may not be able to increase the levels of MnSOD immunoreactive protein or activity (5, 87), TPx activity (29, 30), or Trx protein (29) in response to high O_2 concentrations. Inability to increase AOE activities when challenged with hyperoxia is likely to render these neonates more susceptible to O_2-induced lung injury.

Failure of vascular and alveolar development is recognized as hallmark of the "new BPD" (1). An increasing body of evidence suggests that hypoxia-dependent factors play critical roles in lung development, and that deficiency of these factors during lung development can cause perinatal respiratory distress and death. Hypoxia inducible factors 1 and 2 (HIF-1 and HIF-2, respectively) are examples of such important hypoxia-dependent transcription factors (Fig. 2). HIFs are, in turn, responsible for the expression of other important proteins, such as vascular endothelial growth factor, glycolytic enzymes (hexokinase II), and erythropoietin, among others. Genetic inactivation of HIF-1 or HIF-2 is embryonic lethal in mice (22, 62, 131).

Activation of transcription by HIF-1 is complex (53). HIF-1 is a heterodimer composed of the helix–loop–helix/Per-Arnt-Sim protein HIF-1α and the aryl hydrocarbon nuclear translo-

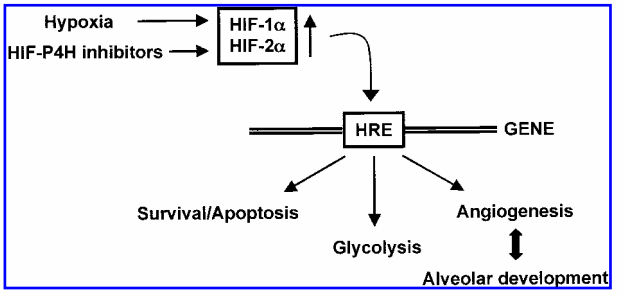

FIG. 2. A schematic diagram of potential effects of HIF stabilization [modified from Carmeliet and Jain (10)]. HIF, hypoxia inducible factor; HRE, hypoxia response element; P4H, prolyl-4-hydroxylase.

cator (ARNT), also known as HIF-1β (73). ARNT functions as a dimerization partner for HIF-1α, and also for several other mammalian proteins. HIF-1α is regulated by cellular O_2 concentration such that levels of HIF-1α protein increase exponentially as O_2 concentration decreases. By contrast, ARNT is expressed independently of the prevailing partial pressure of O_2. Active HIF-1 complex can accumulate in the nucleus, bind to specific DNA sequence(s) (hypoxia response elements), and enhance transcription of hypoxia-inducible genes. Activity of HIF-1 generally is limited by the stability of the HIF-1α subunit. In oxygenated cells replete with iron, HIF-1α is rapidly destroyed. Recently, the mechanisms by which this can occur have been elucidated (60, 63, 74).

Growth of the developing lung normally proceeds in an extremely hypoxic environment. Hence, even 21% O_2 is relative hyperoxia for the developing fetal lung, regardless of the antioxidant status. During fetal development, HIF-1α is expressed at highest levels in the brain, heart, and lungs (79). Premature delivery would be anticipated to cause abrupt degradation of HIFs. Loss of the activities of HIF-1α and HIF-2α may, in turn, lead to failure to synthesize proteins essential for pulmonary vascular growth and, thereby, lead to impaired alveolarization. It follows that therapies which allow stabilization of HIF-1α and HIF-2α and, subsequently, allow HIF-dependent protein expression, could diminish lung injury and improve alveolar and vascular development (Fig. 2). Stabilization of HIF through inhibition of degradation recently has been tested in experimental models (61, 126). Just as hypoxic environment favors the rapid growth of malignant tumors (101), it could also favor the rapid growth of the fetal lung prior to birth. Continued fetal life *ex utero*, by means other than hypoxia, would be expected to allow appropriate expression of hypoxia-dependent factors during critical stages of lung development. Naturally, these approaches may have potential risks and benefits. For example, prolyl-4-hydroxylase inhibitors may be relatively nonspecific, *i.e.,* inhibit collagen synthesis, and thereby be antiproliferative for a variety of cell types, thus aggravating the principal problem of BPD. However, specific stabilization of HIFs may be a desirable goal in the premature neonate at risk for developing BPD.

Conclusions

Solid experimental data from many lesser species support the theory that preterm neonates are deficient in pulmonary antioxidants due to their developmental regulation, such that the activities of these enzymes increase during the final third of gestation. However, considerable interspecies differences have been observed in developmental profiles of various antioxidants. Our results on fetal and neonatal baboons and humans show that coordinated up-regulation of pulmonary AOE activities does not appear to take place during primate ontogeny. However, preterm primate newborns may lack the ability to respond to the rigors of oxidative stress with increased antioxidant profiles, which may contribute to the development of O_2-mediated lung injury. Thus far, clinical trials attempting to prevent BPD or death by treatment with single antioxidant agents have failed, despite advantageous biochemical changes detected. More novel approaches, such as modulation of certain transcription factors, signaling pathways, and/or growth factors, could lead to enhanced expression of several antioxidant and growth-favoring pathways, and thereby might lead to desirable protective effects in the preterm lung under oxidative stress.

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ABBREVIATIONS

AOE(s), antioxidant enzyme(s); AP-1 and AP-2, activator protein 1 and 2, respectively; APE-1, apurinic/apyrimidinic endonuclease 1; ARNT, aryl hydrocarbon nuclear translocator; BPD, bronchopulmonary dysplasia; CAT, catalase; CuZnSOD, copper-zinc superoxide dismutase; ECSOD, extracellular superoxide dismutase; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; HIF-1α and HIF-1β, hypoxia inducible factor 1α and 1β, respectively; HIF-2α, hypoxia inducible factor 2α; HO-1 and HO-2, heme oxygenase 1 and 2, respectively; H_2O_2, hydrogen peroxide; MnSOD, manganese superoxide dismutase; NAC, *N*-acetylcysteine; NF-κB, nuclear factor κB; $^{\cdot}NO$, nitric oxide; $O_2^{\cdot -}$, superoxide anion; RDS, respiratory distress syndrome; ROS, reactive oxygen species; SOD, superoxide dismutase; TPx, thioredoxin peroxidase; TR, thioredoxin reductase; Trx, thioredoxin.

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