Hyperoxia inhibits protein synthesis and increases eIF2α phosphorylation in the newborn rat lung

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Submitted 5 August 2009; accepted in final form 4 February 2010

Konsavage W, Zhang L, Vary T, Shenberger JS. Hyperoxia inhibits protein synthesis and increases eIF2α phosphorylation in the newborn rat lung. Am J Physiol Lung Cell Mol Physiol 298: L678–L686, 2010. First published February 5, 2010; doi:10.1152/ajplung.00262.2009.—Prolonged exposure to hyperoxia contributes to aberrant lung growth in premature infants. Of the deleterious effects induced by hyperoxia, alterations in protein synthesis are likely to be of great importance to the developing lung. Regulation of mRNA translation occurs predominantly at the level of initiation via control of mRNA/ribosome binding by proteins known as eukaryotic initiation factors (eIF). Although hyperoxia is known to suppress mRNA translation in adult lungs, little is known regarding the effects in newborns or the involved mechanism. This study was performed to determine the effect of exposure to 95% O2 on pulmonary protein synthesis in 4-day-old Sprague-Dawley rat pups. We found that hyperoxia suppressed the incorporation of [3H]phenylalanine into lung protein over time, resulting in a 23% reduction after 72 h with pups reared in room air. This effect was preceded by a shift in total lung RNA to lower order polysomes. Hyperoxia increased eIF4G-eIF4E binding, a surrogate maker of eIF4F complex assembly, and initially activated, then suppressed, the phosphorylation of ribosomal S6 kinase 1 and ribosomal S6 protein, downstream targets of mammalian target of rapamycin. Exposure to 95% O2 enhanced the phosphorylation of the translational repressor eIF2α in whole lung extracts and the immunoreactivity of phosphorylated eIF2α in epithelial cells. Cell culture studies further demonstrated that hyperoxia increases eIF2α phosphorylation in lung epithelial cells, but not in lung fibroblasts. These findings illustrate that hyperoxia-induced suppression of mRNA translation in the newborn lung is accompanied by increased phosphorylation of eIF2α in the epithelium.

administration of high concentrations of oxygen (hyperoxia) is a potentially life-saving therapy for the treatment of premature infants with surfactant deficiency. Direct exposure of the immature lung to this atmosphere, however, is potentially injurious. In animal models, hyperoxia severely hinders lung alveolarization and disrupts pulmonary vascularization, alterations that adversely affect pulmonary function and gas exchange (24). In clinical practice, hyperoxia is a well-known contributor to the pathogenesis of bronchopulmonary dysplasia (BPD) and the reduction in alveolar surface area (4). At the cellular level, hyperoxia generates reactive oxygen species capable of influencing vital cellular processes regulating cell division, survival, and gene expression (24). Of the many deleterious effects induced by exposure to hyperoxia, alterations in protein synthesis are likely to be of great importance to the developing lung.

Although hyperoxia-induced suppression of protein synthesis was first reported nearly 40 years ago in cultured cells, few subsequent reports document the phenomenon in intact animals. In adult rat lung slices, exposure to 98% O2 decreases the incorporation of L-[U-14C]leucine into protein after 24 h (9). Kelly (15) defined the ability of hyperoxia to reduce protein synthesis in adult rats, demonstrating a reduction in the fractional rate of protein synthesis, total protein synthesis, and the efficiency of protein synthesis without altering total lung RNA or RNA/protein content. Only a single trial has investigated the effect of hyperoxia on protein synthesis in newborn animals, and this trial failed to identify alterations in the incorporation of leucine into protein after a very brief, 2-h exposure to 100% O2 (32). In effect, the protein synthetic response to hyperoxia in newborn animals remains largely unexplored.

Translation of mRNA into protein is divided into three distinct phases, initiation, elongation, and termination, with initiation being the most highly regulated. More than a dozen proteins known as eukaryotic initiation factors (eIF) participate in the complex regulation of initiation. Two steps in initiation are known to be regulated: the binding of the mRNA to 40S ribosomal subunit to form the 43S preinitiation complex and the binding of the Met-tRNAiMet (initiator methionyl-tRNA) to the 40S ribosomal subunit. All nuclear-encoded eukaryotic mRNAs contain a 7-methyl-GTP cap at the 5'-untranslated region (UTR; Ref. 11). Thus small alterations in eIF4E activity may produce significant changes in the expression of growth-regulatory proteins.

Binding of the Met-tRNAiMet to the 40S ribosomal subunit is mediated by eIF2. When bound to GTP, eIF2 can recruit Met-tRNAiMet to the 40S subunit (11). On recognition of the start codon, GTP is hydrolyzed, releasing eIF2-GDP. To attract Met-tRNAiMet, GTP must be regenerated, a step catalyzed by the guanylate nucleotide exchange factor eIF2B (7). Phosphorylation of the α-subunit of eIF2 on Ser51 stabilizes and inac-
tivates eIF2α-eIF2B-GDP (26). Phosphorylation of the catalytic ε-subunit of eIF2B also regulates eIF2 activity, most commonly in a negative direction (31). Modulation of initiation, therefore, involves two highly regulated independent processes, the assembly of the eIF4F complex on the 5′ mRNA cap and the successful recruitment of the Met-tRNAiMet by eIF2.

Although it is commonly accepted that hyperoxia inhibits pulmonary protein synthesis, direct evidence from animal models is lacking. As translational control is the final common pathway leading to expression of the pulmonary proteome, understanding the regulation of mRNA translation during O2 exposure is likely to be essential to deciphering the pathogenesis of BPD. To gain a more thorough understanding of the impact of hyperoxia on protein synthesis in the lung, we investigated the effect of 95% O2 on pulmonary protein synthesis and the key regulators of initiation in newborn rat pups. Based on our previous work in lung cells, we hypothesized that hyperoxia would reduce pulmonary protein synthesis, hinder the assembly of active eIF4F complexes, and augment eIF2α phosphorylation (28).

METHODS

Animal model and conditions. All animal protocols were approved by the Institutional Animal Care and Use Committee at the Pennsylvania State College of Medicine. Timed-pregnant, Sprague-Dawley rats (day 14 of gestation; Charles River Laboratories, Boston, MA) were housed in standard rat cages, and on day 4 pups/dam combinations were culled to 9–10, and litters were placed into Plexiglas chambers (Small A-Chamber cat. no. A-15274; BioSpherix, Redfield, NY). Dams were supplied with standard rat chow and water ad libitum, exposed to routine day-light cycles of 12 h, and maintained at 26°C and 75–80% humidity. Dams were interchanged daily between libitum, exposed to routine day-light cycles of 12 h, and maintained at 26°C and 75–80% humidity. Dams were interchanged daily between groups to minimize O2 toxicity to the dams. On day 4, chambers were circulated with either room air or 95% O2. Delivery of 100% O2 was continually adjusted using a computerized system (OxyCycler A; BioSpherix) to ensure constant 95% O2. In both chambers, the atmosphere was continually circulated, and CO2 concentrations were monitored. The concentration of CO2 measured by the BioSpherix sensor was maintained <0.5% by adjusting the degree of chamber “leak.” Room air and O2-exposed animals were studied concurrently. Pups treated with rapamycin were injected with 4 mg/kg rapamycin (LC Laboratories, Woburn, MA) in 5% DMSO or an equivalent volume of DMSO vehicle 1 h before placement in their respective atmosphere.

Tissue collection and preparation. After exposure, pups were weighed and killed by decapitation. A midline thoracotomy was performed, and the lungs were removed en bloc. Lungs were frozen in liquid N2 and stored for later analysis. Dry left lung weights were obtained by drying tissue in an oven at 48°C. Lungs were weighed hourly, and the final weight was selected when <5 mg change in weight was obtained on successive measurements (6 h in all cases).

Fractional global protein synthetic rates. Fractional global protein synthetic rates were determined by a modified version of the method described by Garlick et al. (10, 30). Briefly, pups were weighed and injected with a flooding dose of 1-[1-14C]phenylalanine ([14C]Phe; 100 µCi/10 g body wt; 150 mmol/l containing 100 µCi/ml; GE Scientific, Piscataway, NJ). Labeled pups were placed into a separate, warmed container in their respective atmosphere. After 15 min, pups were decapitated, and blood was collected for determination of serum-specific activity of Phe. Blood was centrifuged at 13,000 g for 1 min at 4°C, and plasma was collected. The lungs were flash-frozen with liquid N2 for later analysis.

The specific radioactivity of Phe (PheSR) in the serum was measured using HPLC as previously described (6). Serum was deproteinized with 10% trichloroacetic acid, the pH was adjusted to 9.0, and an aliquot was derivatized with 4-dimethylaminobenzene-4′-sulfonyl chloride. Serum supernatant was chromatographically separated using an Alltech Econosphere C18 (5 μm) column with an in-line Beckman 450 HPLC equipped with a Beckman 171 radioisotope detector (Beckman Coulter, Fullerton, CA). The PheSR was calculated by dividing the dpm of the Phe peak by the concentration of Phe in the sample (6).

Incorporation of [14C]Phe into lung protein was measured in both lungs homogenized in 7 volumes of ice-cold 0.9% NaCl (wt/vol) using a Polytron tissue homogenizer. Total lung protein was precipitated by the addition of 2 volumes (vol/vol) of 10% trichloroacetic acid. Homogenates were then cleared by centrifugation, and the resulting pellet was rinsed three times with 10% trichloroacetic acid (30). The remaining pellet was dissolved in 2-ml 1 N NaOH for 1 h at 37°C. An aliquot of the homogenate was taken to measure protein concentration via the biuret method. The radioactivity was counted by liquid scintillation, and rate of protein synthesis was estimated from the rate of incorporation of radioactive Phe into lung protein using the PheSR of serum as representative of the precursor pool (6). Previous investigations have illustrated that aminoacyl-tRNA and blood- and tissue-free amino acid pools are rapidly equilibrated after administration of flooding doses of Phe (5). Accordingly, the rate of protein synthesis [nmol Phe/(mg protein/h)] can be calculated from the equation:

\[
\text{Protein synthesis} = \frac{\text{dpm of tissue protein}(\text{PheSR} \times t)}{\text{dpm of tissue protein}}
\]

where \( t \) equals the elapsed time (h) after injection of [14C]Phe. To obtain the tissue protein synthesis in the lung, protein synthesis was normalized to wet lung weight.

Polysome analysis. Sucrose gradient centrifugation was employed to analyze lung polysome aggregation. Lung tissue was homogenized for 20 s in 10 volumes (wt/vol) of resuspension buffer [50 mM HEPES, pH 7.4, 75 mM KCl, 5 mM MgCl2, 250 mM sucrose, 100 µg/ml cycloheximide, 2 mM DTT, 1% Triton X-100, 1.3% deoxycholate, 10 µl/ml SUPERasin (Ambion, Austin, TX)] using a Polytron. Homogenates were incubated on ice for 5 min, and then 150 µl/ml Tween-deoxycholate (1.34-ml Tween 20, 0.66-g deoxycholate, 24 13.2 72 18.7 360 8.9

Table 1. Effect of hyperoxia on body and lung weights

<table>
<thead>
<tr>
<th>Time, h</th>
<th>RA Body Weight, g</th>
<th>RA Left Dry Lung Weight, mg</th>
<th>RA Wet-to-Dry Left Lung Ratio</th>
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<td>72</td>
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Values are means ± SE for body weight (n = 9), dry lung weights (n = 5), and wet-to-dry lung weight ratios (n = 5). RA, room air; OX, 95% oxygen. Time 0 represents animals at the start of exposure. ANOVA indicated a time-dependent increase in body weight and dry lung weights. Exposure to 95% O2 failed to alter dry lung weights but decreased the wet-to-dry ratio at 24 h (*P < 0.05).
Fig. 1. Effect of room air (●) and 95% O₂ (○) on pulmonary protein synthesis. Sprague-Dawley rat pups were exposed to room air or 95% O₂ for 24–72 h, and pulmonary protein synthetic rates were normalized to precipitated lung protein. Values represent means ± SE (n = 8–27). Data were analyzed by 2-way ANOVA with Fisher post hoc testing. A significant effect of hyperoxia was identified (P < 0.05) with an individual difference identified at 72 h (**P < 0.05). Phe, phenylalanine.

Fig. 2. Impact of hyperoxia on polysome aggregation. Total lung RNA was subjected to sucrose density gradient centrifugation as described under METHODS. Tracings represent the relative quantity of RNA at specific sucrose density as measured by optical density (OD) 254 nm. The black arrow represents the 80S monomer peak. The dashed lines denote nonpolysomal (NP) and polysomal (P) RNA. The mean ratio of P/NP for room air- (black) and 95% O₂-exposed (gray) animals is represented by columns (n = 7–8). Bars represent SE. The P/NP ratio for room air-exposed pups did not change over time. A significant effect of hyperoxia on polysome distribution was identified by ANOVA (P < 0.05). *Differences between groups after post hoc analysis.

18-ml sterile H₂O (©) was added, and the samples were mixed. Samples were then cleared by centrifugation, supernatants were layered onto 20–47% linear sucrose gradients, and gradients were centrifuged at 90,000 g for 4 h at 4°C in a Beckman SW 28 rotor. After centrifugation, each gradient was upwardly displaced using a Fluorinert fractionator, and fractions were read through a spectrophotometer. The optical density at 254 nm was recorded by an attached chart recorder.

Determination of eIF4F assembly. Affinity chromatography was used to separate eIF4E containing protein complexes as previously described (28). Briefly, frozen left lungs were pulverized using an ice-cooled, Dounce tissue homogenizer. Pulverized tissue was dissolved in 1 ml of CHAPS lysis buffer (40 mM HEPES, pH 7.5, 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM β-glycerophosphate, 40 mM NaF, 1.5 mM sodium orthovanadate, 0.1 mM PMSF, 1 mM benzamidine, 1 mM DTT, 0.3% CHAPS). One hundred micrograms of total lung protein was combined with 100 μl of washed m7GTP-Sepharose beads (GE Scientific) in buffer A (20 mM Tris, pH 7.4, 0.2 mM EDTA, 100 mM KCl, 7 mM 2-mercaptoethanol) and incubated for 2 h at 4°C with gentle agitation. Following incubation, beads were pelleted and washed, and the protein complexes were removed by boiling. Affinity-purified protein complexes were separated by electrophoresis and immunoblotted with antibodies to eIF4E, eIF4G, and 4E-BP1 as described below. Immunoblots were visualized by chemiluminescence, band density was quantified, and expression was normalized to β-actin for each gel. Blots for phosphorylated proteins were not stripped and reprobed due to interference of several of the phosphorylation-specific antibodies with the total protein antibodies. Accordingly, each gel was normalized to β-actin for that gel, and the ratio of phosphorylated to total protein was calculated as (phospho/actin)/(total/actin) using densitometry. Because of the large number of animals and conditions, gels representing different time points were run separately and combined in the figures.

Immunofluorescence microscopy. For immunofluorescence microscopy, lung lobes were frozen in liquid nitrogen, embedded in optimum cutting temperature compound (OCT), and cut into 6-μm sections. Sections were stained with rabbit anti-eIF2α (Ser51; Abcam, Cambridge, MA) at a concentration of 1:50 in 0.25% BSA/TBS overnight. After rinsing in TBS, sections were incubated with donkey anti-rabbit Cy3-labeled secondary antibody (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA) in 0.25% BSA/TBS for 1.5 h. After rinsing, sections were viewed at ×10–40 using a Olympus fluorescence microscope. A minimum of three sections from three animals in each group were examined.

Cell culture. To determine the effect of hyperoxia on individual human lung cell types, we exposed two epithelial and fibroblast cell lines to room air or hyperoxia for 24 h using a BioSpherix OxyCycler C designed for cells. A549 cells represent a type II cell-like adenocarcinoma cell line, whereas NCL20 cells are simian virus 40 (SV40)-immortalized bronchial epithelial cell line (25). The fibroblast cells were derived from neonatal human lung fibroblasts (HLF) and fetal lung fibroblasts (WI-38) (28). All cells were propagated in DMEM supplemented with 10% FBS, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 2 mM glutamine. Cells were plated at a density of 20,000/cm² and allowed to attach overnight before exposure to room...
air or 95% O₂-5% CO₂ for 24 h. After exposure, cells were rinsed in cold PBS and lysed in RIPA buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 0.5% Nonidet P-40, 1 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF, and EDTA-free protease inhibitor cocktail (Roche, Branford, CT)]. Protein assays and immunoblotting were performed in a manner analogous to that for whole lung lysates.

Statistical analysis. Animal trials were conducted a minimum of three times using pups from at least three different litters, with the exception of the rapamycin experiment that was conducted using four animals in each group from two scrambled litters. For cell culture, three individual experiments were performed for each cell line. For all animal experiments, the effects of time, atmosphere, and potential interactions were tested using ANOVA with Fisher protected least significant differences post hoc analysis to identify individual differences. All data are listed as means ± SE with the level of significance set at $P < 0.05$.

RESULTS

Hyperoxia does not alter body or wet lung weight. All animals in both the room air and 95% O₂ groups survived the 3-day exposure period. There was no identifiable change in pup nursing behavior during exposure, and all pups undergoing laparotomy contained milk within the stomach indicating that they were postprandial during the measurements. Hyperoxia had no effect on total body weight in serially weighed animals (Table 1). Although left lung dry weights were similar between groups, wet-to-dry left lung ratios did show a significant reduction in the hyperoxia group but only at 24 h (Table 1).

Hyperoxia inhibits pulmonary protein synthesis and polysome aggregation. As shown in Fig. 1, hyperoxia reduced the rate of pulmonary protein synthesis over time (ANOVA, $P < 0.05$) with the 23% reduction at 72 h identified as significant on post hoc analysis. To delineate whether alterations in the initiation or elongation phase of translation mediate hyperoxia-induced reductions in pulmonary protein synthesis, we examined polysome profiles from total lung RNA. As expected, total RNA in the lung homogenates increases in both groups over time as demonstrated by the upward shift in OD values (Fig. 2). Fractionation of total RNA through 20–47% sucrose gradients demonstrated that hyperoxia shifts the polysomal distribution toward faster migrating or lower order polysomes, indicative of an inhibition of mRNA translation at the initiation phase of protein synthesis (Fig. 2). In contrast, the polysomal-to-non-polysomal ratio in room air-treated animals did not change significantly from 24 to 72 h. The rapid alteration in polysome profiles suggests that polysome aggregation may be a more sensitive indicator of changes in mRNA translation than total protein synthetic rates.

Hyperoxia augments eIF4F assembly. Nutrients and growth factors increase the phosphorylation of 4E-BP1, releasing eIF4E to bind eIF4G. In general, maximal translation rates coincide with enhanced eIF4G-eIF4E binding, a surrogate for the increased assembly of active eIF4F complexes. Affinity purification of cell lysate with mGTP-coated beads allows for the identification of the relative binding of eIF4E to both eIF4G and 4E-BP1. With this approach, we found that hyperoxia augmented the binding of eIF4E with eIF4G at both 24 and 72 h (Fig. 3A). On each day, hyperoxia increased the relative binding of eIF4E with eIF4G twofold. Investigation of the binding of eIF4E with the repressor protein, 4E-BP1, did not denote significant alterations by hyperoxia but did identify an interaction between hyperoxia and time, such that 4E-BP1-eIF4E binding of eIF4E with eIF4G twofold. Investigation of the binding of eIF4E with the repressor protein, 4E-BP1, did not denote significant alterations by hyperoxia but did identify an interaction between hyperoxia and time, such that 4E-BP1-eIF4E binding was greater at 24 h in hyperoxia-treated animals but less at 72 h ($P < 0.0001$ by ANOVA; Fig. 3B).

In summary, these results indicate that hyperoxia promotes the assembly of eIF4F complexes despite an overall reduction in protein synthetic rates.
Hyperoxia alters the phosphorylation of mammalian target of rapamycin substrates influencing translation. Given that eIF4F complex assembly is influenced by changes in the phosphorylation state of 4E-BP1, we next chose to examine the effect of hyperoxia on specific 4E-BP1 phosphorylation sites known to modulate eIF4E binding. Activation of mammalian target of rapamycin (mTOR) complex 1 (mTORC1, mTOR-GβL-raptor) stimulates the phosphorylation of 4E-BP1 beginning with Thr^{37/46}, then Thr^{70}, and finally Ser^{65} (21). Phosphorylation of Thr^{37/46} and Thr^{70} appears to be required for the dissociation of eIF4E from 4E-BP1, whereas phosphorylation on Ser^{65} site has been postulated to inhibit the rebinding of eIF4E to 4E-BP1 (21). The hierarchical nature of the phosphorylation produces two bands for rat 4E-BP1 such that total protein and the “priming” phosphorylation at Thr^{37/46} yield two bands, whereas later phosphorylation at Thr^{70} and Ser^{65} produces a single band. Immunoblotting of whole lung lysates revealed that hyperoxia had no effect on the phosphorylation at Thr^{37/46} but reduced 4E-BP1 phosphorylation on Thr^{70} by 65% at 72 h (Fig. 4). We were unable to consistently indentify Ser^{65} phosphorylation in either room air or oxygen-exposed rat pups.

Because the findings for Thr^{70} are in opposition to those observed for 4E-BP1-eIF4E binding, we next sought to investigate the phosphorylation of an additional mTORC1 substrate known to influence translational efficiency, S6K1. Phosphorylation of S6K1 by mTORC1 occurs in a canonical manner like 4E-BP1 and enhances the phosphorylation of the 40S subunit S6Rp, an event correlating with increased ribosome biogenesis and protein synthesis (3). Accordingly, we studied the impact of hyperoxia on the phosphorylation of S6K1 on Thr^{389} (mTORC1 site) and S6Rp on Ser^{235/236}. As shown in Fig. 4, hyperoxia increased the phosphorylation of S6K1 and S6Rp at 24 h by 3.5- and 2-fold, respectively. By 72 h, however, the effect of hyperoxia on S6K1 and S6Rp phosphorylation was markedly diminished such that S6Rp phosphorylation was less than in room air-exposed pups. This effect was apparent through the detection of a significant interaction between hyperoxia and time for each phosphoprotein (ANOVA, P < 0.0001). Cumulatively, these observations indicate that the initial activation of mTORC1 substrates is reversed during prolonged exposure.

Fig. 4. Effect of 95% O2 on phosphorylation of mammalian target of rapamycin (mTOR) complex 1 (mTORC1) substrates. A: immunoblots are representative of the relative phosphorylation of ribosomal S6 kinase 1 (S6K1), ribosomal S6 protein (S6Rp), and 4E-BP1. B: histograms show the mean phosphorylation (p-) in the lungs of room air- (black) and 95% O2-exposed (gray) pups. Columns represent means (n = 11–17), and bars represent SE. There was no effect of hyperoxia or time on 4E-BP1 phosphorylation on Thr^{70} by 65% at 72 h (Fig. 4). We were unable to consistently indentify Ser^{65} phosphorylation in either room air or oxygen-exposed rat pups.

A significant interaction between hyperoxia and time was also identified for S6K1, S6Rp, and 4E-BP1 (Thr^{70}) (ANOVA, P < 0.001). *Differences between groups after post hoc analysis.
Hyperoxia increases the phosphorylation of eIF2α. Assembly of the eIF4F complex is only one aspect of mRNA translation under regulatory control. Recruitment of the initiator Met-tRNA<sub>Met</sub> to the 40 S ribosomal subunit during the initiation phase of translation is catalyzed by the heterotrimERIC factor, eIF2. To attract Met-tRNA<sub>Met</sub>, eIF2 must bind GTP. This step is accomplished by eIF2B, a guanylate nucleotide exchange factor comprised of three regulatory and two catalytic subunits of which the ε-catalytic subunit is required for full activity (31). Phosphorylation of eIF2Be on Ser<sup>535</sup> inhibits eIF2 activity (31). Likewise, phosphorylation of the α-subunit of eIF2 on Ser<sup>51</sup> converts eIF2α from a substrate to an inhibitor, effectively stabilizing and inactivating eIF2α-eIF2B-GDP (26). Accordingly, relatively small changes in eIF2α phosphorylation can have a dramatic impact on translational efficiency. Analysis of eIF2Bε in whole lung lysates failed to identify a consistent effect of hyperoxia on eIF2Bε phosphorylation (Fig. 5). Hyperoxia did, however, increase the phosphorylation of eIF2ε on Ser<sup>51</sup>, an effect that was observed at both 24 and 72 h (Fig. 5).

**mTOR inhibition does not alter hyperoxia-mediated eIF2α phosphorylation.** To evaluate a potential connection between the apparent increase in mTORC1 activity and eIF2α phosphorylation in whole lung lysates, we treated animals with the mTOR inhibitor rapamycin 1 h before exposure to 95% O<sub>2</sub>. All animals tolerated the injections of vehicle and rapamycin well, with no discernable differences in behavior. Rapamycin markedly decreased S6K1 and S6Rp phosphorylation in whole lung extracts of both room air- and oxygen-exposed pups (Fig. 6). In contrast, rapamycin had no effect of hyperoxia-induced increases in eIF2α phosphorylation compared with noninjected or vehicle-injected. Interestingly, rapamycin did tend to increase eIF2α phosphorylation in room air-breathing pups, perhaps indicating that under basal conditions, inhibition of mTOR provides sufficient stress to enhance eIF2α phosphorylation (Fig. 6).

**Cell-specific alterations in eIF2α phosphorylation.** Although our findings indicate that hyperoxia-induced reductions in protein synthesis correlate with increased eIF2α phosphorylation in whole lung homogenates, they cannot identify particular cell populations involved. Accordingly, we used immunofluorescence microscopy to “screen” for cell-specific alterations in eIF2α phosphorylation. Examination of sections from room air- and hyperoxia-exposed pups revealed that hyperoxia increased eIF2α phosphorylation in airway epithelial cells and, to a lesser extent, in scattered cells throughout the lung parenchyma (Fig. 7). To provide further evidence for the effect of hyperoxia on various lung cell populations, we exposed two human lung epithelial and fibroblast cell lines to 24 h of 95% O<sub>2</sub>. As shown in Fig. 8, hyperoxia increased eIF2α phosphorylation in each of the epithelial cell lines, but not in the fibroblast cell lines. These data support the microscopic findings that hyperoxia-induced alterations in eIF2α phosphorylation occur in epithelial cells within the lung.

**DISCUSSION**

The immature lung is particularly vulnerable to the toxic effects of high concentrations of inspired O<sub>2</sub> secondary to the structural immaturity of the parenchyma, impaired antioxidant...
capacity, and increased susceptibility to infection (24). The cumulative effects of hyperoxia hinder lung growth and development by disrupting cellular differentiation, increasing cell death, and altering gene expression. Translation of mRNA is the final common pathway whereby the expression of human genome is regulated, indicating that effects of hyperoxia on protein synthesis are likely to influence the histopathology of O2-induced lung injury and BPD. This report shows that hyperoxia modestly inhibits pulmonary protein synthesis and increases the phosphorylation of eIF2α in the lung while transiently augmenting the activity of mTOR, thereby illustrating the complexities of the hyperoxic response. These findings are summarized in Fig. 9.

More than three decades have passed since Gacad and Massaro (9) first documented a 25% decrease in the incorporation of amino acids into protein in cultured lung slices obtained from hyperoxia-exposed male rats. A subsequent study identified a 25% reduction in the fractional protein synthetic rate of intact adult rat lungs exposed to hyperoxia for 24 h (15). We expanded on these previous reports by illustrating that exposure of nursing newborn rats to hyperoxia reduces the pulmonary protein synthetic rate in a time-dependent fashion, culminating in a similar 23% reduction after 72 h. This reduction in pulmonary protein synthesis is preceded by a transient shift in the distribution of polysomes from heavier to lighter fractions, consistent with inhibition of mRNA translation during initiation. Normalization of polysome profiles at 72 h implies that prolonged O2-exposure slows initiation and elongation rates similarly and that the combined effects reduce the overall protein synthetic rate. It is also interesting to note that changes in pulmonary protein synthesis are not reflected in the dry lung weights. Given the magnitude of the change in synthetic rates, longer durations of exposure may be necessary to produce significant reductions in dry lung weight. Hyperoxia-induced inhibition of mRNA translation at the level of initiation in the newborn lung correlates well with our (28) previous work in human neonatal lung fibroblasts. In that study, early reductions in global protein synthesis corresponded to inhibition of 4E-BP1 phosphorylation and increased 4E-BP1 binding with eIF4E, suggesting that hyperoxia diminishes assembly of active eIF4F complexes (28). In newborn rat lungs, however, hyperoxia reduces global protein synthesis while fostering eIF4F assembly. Although hyperoxia increases eIF4G-eIF4E binding within 24 h, reciprocal decreases in 4E-BP1-eIF4E do not appear until 72 h. As noted previously, the binding of 4E-BP1 to eIF4E is inversely regulated by the degree of phosphorylation. To the contrary, changes in 4E-BP1 phosphorylation do not correlate with changes in 4E-BP1 binding in O2-exposed pups. These findings suggest that hyperoxia may alter Ser65 phosphorylation and influence the reassociation of 4E-BP1 with eIF4E or the expression and phosphorylation of one of the other 4E-BP isoforms (21).

The ability of hyperoxia to reduce 4E-BP1-eIF4E and increase the phosphorylation of S6K1 and S6Rp are also consistent with the activation of mTORC1. Exposure of lung epithelial cells to hyperoxia activates Akt, an upstream kinase in the mTORC1 signaling pathway (19). Although hyperoxia-in-
duced Akt activity may initially stimulate mTORC1 activity in the lung, such changes are transient as continued exposure inhibits the phosphorylation of S6K1, S6Rp, and 4E-BP1 by 72 h. In this regard, stimulation of mTORC1 activity may have beneficial effects aside from the maintenance of protein synthesis. The observation that constitutive Akt activation in hyperoxia-exposed lung microvascular endothelial cells increases glucose utilization and reduces cytotoxicity is highly suggestive of such a process (1).

Phosphorylation of eIF2α inhibits the guanylate nucleotide exchange factor eIF2B and the recruitment of the initiator Met-tRNA<sup>Met</sup> to the 40S ribosome (2). Because phosphorylation increases the affinity of eIF2-GDP for eIF2B by 150-fold, small increases in phosphorylated eIF2α markedly inhibit cap-dependent mRNA translation (23). Four stress-responsive kinases are currently known to phosphorylate eIF2α: heme-regulated inhibitor (HRI), general control nonderepressible 2 (GCN2), double-stranded RNA-dependent kinase (PKR), and PKR-like endoplasmic reticulum kinase (PERK; Refs. 16, 33). Original descriptions of each kinase reflect the nature of the stress response: HRI to heme/iron deficiency, GCN2 to amino acid starvation, PKR to viral infection, and PERK to endoplasmic reticulum stress (16). Reactive oxygen or nitrogen species have been shown to activate each of the eIF2α kinases under various conditions (12, 20, 29). Increased phosphorylation of eIF2α may also result from inactivation of either or both eIF2α phosphatases, growth-arrest and DNA damage-inducible protein-34 (GADD34) and constitutive repressor of eIF2α phosphorylation (CREP), proteins that form eIF2α-specific phosphatase complexes with protein phosphatase 1 (14). Although hyperoxia enhances eIF2α phosphorylation on Ser<sup>51</sup> by 40–100% in the lungs of newborn rats, the concurrent reductions in protein synthetic rates may not be causal. Future studies that genetically manipulate eIF2α kinases and phosphatases will be necessary to draw more definitive conclusions.

Despite the ability of phosphorylated eIF2α to suppress cap-dependent mRNA translation, the net effect on all mRNAs is not likely to be identical. Some mRNAs, due to unique sequences within the 5′-UTR, are translationally upregulated. In particular, those possessing upstream open reading frames (ORF) such as activating transcription factor-4 (ATF4) and cationic amino acid transporter-1 are preferentially expressed during periods of stress (8, 13). At least one report indicates that ATF4 interacts positively with the zinc-finger transcription factor, NF-erythroid 2-related factor-2 (Nrf2; Ref. 13). On translocation to the nucleus, Nrf2 binds the promoters of NAD(P)H-quinone oxidoreductase, glutathione S-transferases, and heme oxygenase 1, leading to increased expression of these important antioxidant enzymes (13). Hyperoxia-induced Nrf2 protein expression has been found to be cytoprotective in type II alveolar epithelial cells and to ameliorate O<sub>2</sub>-induced lung injury in mice (18). Accordingly, the integrated activation of mTOR and eIF2α phosphorylation during hyperoxia may be adaptive. The ability of rapamycin to inhibit S6K1 and S6Rp phosphorylation without influencing eIF2α phosphorylation during hyperoxia may be indicative of the two events are independent. Together, the signaling pathways may serve to increase energy stores, reduce energy consumption, and promote the expression of factors necessary for survival in an O<sub>2</sub>-enriched environment.

Alternatively, the discovery that hyperoxia alters distinct translational regulatory pathways in opposing directions might simply reflect unique responses in multiple cell types. A weakness of examination of whole lung extracts is the potential to obscure effects in small fractions of cells among the ∼40 known populations in the lung (17). Fluorescence microscopy, coupled with cell culture, identifies lung epithelial cells as one target of hyperoxia-induced alterations in translational signaling. The same studies reveal little change in eIF2α phosphorylation in O<sub>2</sub>-exposed lung fibroblasts, indicating that hyperoxia does not modulate mRNA translation in all cell types or via the identical signaling pathway. Application of similar approaches to other lung cell types, particularly endothelial cells, macrophages, and type I/II alveolar epithelial cells, are necessary to delineate the contribution of translation regulation to aberrations in lung development mediated by exposure to high concentrations of O<sub>2</sub>. Finally, because the cell culture studies were not performed in cells derived from newborns, we cannot exclude the possibility that hyperoxia-induced eIF2α...
phosphorylation is age-dependent. Nonetheless, we have observed increases in phosphorylation in the lungs of 30-day-old rats and adult mice (data not shown), suggesting that eIF2α phosphorylation is unlikely to be an age-specific signaling event.

In summary, this study demonstrates that hyperoxia reduces global pulmonary protein synthesis in the newborn lung in association with alterations in signaling pathways regulating translation initiation. The findings further reveal that lung epithelial cells, particularly within the airway, are susceptible to O2-induced eIF2α phosphorylation. Determination of whether such changes are adaptive, deleterious, or integral to the suppression of mRNA translation awaits further study.

ACKNOWLEDGMENTS

We acknowledge the expert technical assistance of Gina Deiter, Rachel L. Fogel, and Shane Miller in the performance of the protein synthesis assays.

GRANTS

This project was supported by a grant to J. S. Shenberger from the Children’s Miracle Network at the Milton S. Hershey Medical Center.

DISCLOSURES

No conflicts of interest are declared by the author(s).

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