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ISSN 2470-0983

Pediatrics and Neonatal Nursing: Open Access

Research Article | Volume: 2.3 | Open Access

Maintenance of Feeding-Induced Translation Initiation Complex Assembly in the Lungs of Hyperoxia-Exposed Newborn Rats

Wesley M Konsavage[1], Bethany Edwards[2] and Jeffrey S Shenberger[3]*

[1]*Department of Medicine, Pennsylvania State College of Medicine, Hershey, PA, USA*
[2]*Department of Family Medicine, University of Pittsburgh, Pittsburgh, PA, USA*
[3]*Department of Pediatrics, Baystate Children's Hospital, Springfield, MA, USA*

***Corresponding author:** Jeffrey S Shenberger, Department of Pediatrics, Baystate Children's Hospital, Springfield, MA, USA, Tel: 413-794-5370; **E-mail:** Jeffrey.ShenbergerMD@baystatehealth.org

Received date: 27 May 2016; **Accepted date:** 06 Jun 2016; **Published date:** 10 Jun 2016.

Citation: Konsavage WM, Edwards B, Shenberger JS (2016) Maintenance of Feeding-Induced Translation Initiation Complex Assembly in the Lungs of Hyperoxia-Exposed Newborn Rats. Pediatr Neonatal Nurs Open Access 2(3): doi http://dx.doi.org/10.16966/2470-0983.114

Abstract

Growth failure is common among preterm infants with bronchopulmonary dysplasia (BPD). Hyperoxia, an etiologic factor for BPD, reduces pulmonary protein synthesis in continually nursing rat pups. To determine if hyperoxia-induced alterations in nutrient-sensitive translational regulatory kinase, mTOR (mammalian target of rapamycin) contributes to diminished initiation events, we examined the meal-stimulated mTOR activity and eukaryotic initiation factor 4F (eIF4F) assembly in four-day-old Sprague Dawley rats pups exposed to 24 hours of room air (RA) or 95% O_2 (Ox). At 16 hours, pups were fasted by maternal separation for 8 hours and then fed cow's milk-based formula or water. Fasting suppressed the phosphorylation of the mTOR substrate S6K1 in both RA and Ox animals, but meal-feeding had little effect in either group. While fasting failed to alter eIF4F assembly, feeding increased eIF4F assembly in both groups. Ox attenuated polysome aggregation during initiation under both fasting and fed conditions. Ox increased eIF2α phosphorylation, an mTOR-independent repressor of initiation, 60 minutes after feeding. These findings illustrate that hyperoxia has little negative effect on mTOR-mediated regulation of translation initiation and highlight eIF2α as a potential factor responsible for the diminished protein synthetic capacity of the O_2-treated lung.

Keywords: Bronchopulmonary dysplasia; Hyperoxia; Translation; Phosphorylation

Abbreviations: RA: Room Air; Ox: 95% oxygen; mTOR: Mammalian Target of Rapamycin; BPD: Bronchopulmonary Dysplasia; FSR: Fractional Rate of Protein Synthesis; eIF: Eukaryotic Initiation Factor; Met-tRNA$_i^{Met}$: Initiator Methionyl-tRNA; 4E-BP1: eIF4E-Binding Protein-1; S6Rp: Ribosomal Protein S6; S6K1: Ribosomal Protein S6 Kinase-1.

Introduction

Despite advances in neonatal nutritional management, somatic and tissue-specific growth deficiencies remain a common finding in premature infants diagnosed with bronchopulmonary dysplasia (BPD), a form of neonatal chronic lung disease characterized by a reduction in alveolar surface area and aberrant capillary development [1]. The altered pulmonary architecture in BPD leads to diminished pulmonary function, which may persist into childhood and potentially throughout adult life [2]. During the treatment of respiratory distress syndrome in preterm newborns, exposure to high inspired oxygen concentrations is potentially life-saving. Nevertheless, hyperoxia, the administration of oxygen at a higher partial pressure than is necessary to meet tissues and organs needs, is well-established for its capacity to hinder lung growth in newborn animals and for its etiologic role in the development of BPD [3,4]. Free radicals and reactive species generated by hyperoxia have the capacity to modulate vital cellular metabolic functions which influence growth, including the translational control of gene expression. Within the adult rodent lung, hyperoxia reduces the fractional rate of protein synthesis (FSR) and the efficiency of protein synthesis without altering total lung RNA or RNA/protein content [5]. In the newborn rat lung, hyperoxia suppresses global mRNA translation at the level of initiation and alters the activity of eukaryotic initiation factors (eIF) that regulate mRNA/ribosome binding [6].

Translation of mRNA into protein begins with initiation, which entails binding of the mRNA to 40S ribosomal subunit to form the 43S pre-initiation complex, scanning of the ribosome complex to the start codon, and binding of the Met-tRNA$_i^{Met}$ (initiator methionyl-tRNA) to the 40S ribosomal subunit. The 7-methyl-GTP cap at the 5' terminus of all nuclear-encoded eukaryotic mRNAs binds the 40S ribosomal subunit to the mRNA *via* the eIF4F complex, a heterotrimeric aggregation of the cap-binding protein, eIF4E, the scaffolding protein, eIF4G, and the RNA helicase, eIF4A [7]. Regulation of binding of eIF4E to the 5'-cap is repressed by eIF4E-binding protein-1 (4E-BP1). Phosphorylation of 4E-BP1 by the nutrient-sensitive kinase, mammalian target of rapamycin (mTOR), releases eIF4E, allowing for the formation of active eIF4G:eIF4E complexes [8]. Binding of the Met-tRNA$_i^{Met}$ to the 40S ribosomal subunit is mediated by eIF2 [7]. In order to attract Met-tRNA$_i^{Met}$, eIF2 must generate GTP, a step catalyzed by the guanylate nucleotide exchange factor eIF2B [9]. Phosphorylation of the α subunit of eIF2 on Ser^{51} stabilizes the inactive, GDP-bound, form of eIF2B [10]. The activity of eIF2B is also mediated by its catalytic ε subunit, which is regulated by expression and glycogen synthase kinase-3-mediated phosphorylation [11]. In summary, translational regulation of initiation involves both the assembly of the eIF4F complex on the 5'-mRNA cap and the successful recruitment of the Met-tRNA$_i^{Met}$ by eIF2.

During the newborn period, rapid growth corresponds to high rates of protein synthesis. Accretion of skeletal muscle mass in neonates directly relates to the heightened protein synthetic response to feeding [12]. Feeding also increases the FSR in the newborn lung, but to a lesser extent than in muscle [13]. Mature milk, colostrum, and formula feedings nearly double FSR in the lung of colostrum-deprived, 4-6 day-old piglets [13].

In skeletal muscle, meal-feeding stimulates polysome assembly, eIF4F assembly (increased eIF4G:eIF4E binding), and the phosphorylation of 4E-BP1and ribosomal protein S6 (S6Rp) within 30 minutes [14]. Pathologic conditions have also been shown to negatively influence the translational regulation of protein synthesis. Lipopolysaccharide decreases FSR in glycolytic muscle concomitant with decreases in eIF4G:eIF4E binding and the phosphorylation mTOR substrates, 4E-BP1 and ribosomal protein S6 kinase-1 (S6K1), suggesting that a reduction in mTOR activity is at least partially responsible for the reductions in protein synthesis [15].

We have previously shown that exposure to 95% O_2 reduces protein synthesis and polysome aggregation while simultaneously increasing eIF2α phosphorylation in the lungs of continuously nursing neonatal rats [6]. To directly evaluate the ability of hyperoxia to alter feeding-induced protein synthesis, we designed the current study to test the hypothesis that hyperoxia does not alter feeding-induced initiation complex assembly or mTOR-mediated regulation of mRNA translation in the lungs of newborn rat pups.

Methods

Animal model and conditions

Timed-pregnant, Sprague Dawley rats at day 14 of gestation, (Charles River Laboratories, Boston, MA) were housed in standard rat cages until day 4 after delivery. Littermates were scrambled between dams and the total number of pups culled to 12/ litter. On day-of-life 4, pups were weighed and litters placed into Plexiglas chambers circulated with either room air (RA) or 95% O_2 (Ox) as previously described [6]. Delivery of 100% O_2 was continually adjusted using a computerized system (BioSpherix Ltd., OxyCycler A). In both chambers, the atmosphere was circulated *via* a small fan and CO_2 maintained at <0.5%. Room air and O_2-exposed animals were studied concurrently. Dams were supplied with standard rat chow and water *ad libitum,* exposed toroutine day/light cycles of 12 hours, and maintained at 26°C and 75-80% humidity.

After 16 hours of exposure to either RA or Ox, pups were weighed and separated into 2 groups. In group one, animals were returned to the dam for a total of 24 hours in either RA or Ox (dam-fed, n=3 per atmosphere). The remaining pups were separated from the dam to induce fasting and placed into separate cages (n=9 per atmosphere) lined with standard rodent bedding covering a water-jacketed warming blanket set at 37° C (Gaymar, Orchard Park, NY). Preliminary studies using the phosphorylation of S6K1 in the lungs as an indicator of mTOR activity revealed that 8 hours of separation was required to reduce S6K1 phosphorylation. After fasting, pups were fed 0.4 ml of sterile water (water-fed, n=3 per atmosphere) or human infant premature formula (24 kcal/oz) using a 24 gauge feeding needle attached to a syringe (formula-fed, n=6 per atmosphere). The 0.4 ml volume was determined as the approximate volume of the newborn stomach based upon analysis of dam-fed pups. After feeding, pups were placed back into their respective chamber. Water-fed animals were studied after 30 minutes while half of the formula-fed pups were studied at 30 minutes and half at 60 minutes. At these intervals, pups were sacrificed by decapitation and lungs harvested as described [6]. In a subset of litters, the right gastrocnemius muscle and the entire liver were also harvested. To examine residual mTOR activity after fasting, a group of water-fed pups were treated with rapamycin [4 mg/kg, i.p. (LC Laboratories (Woburn, MA)] in 5% DMSO or an equivalent volume of DMSO vehicle 1 hour prior to placement in their respective atmosphere. Animal protocols were approved by the Institutional Animal Care and Use Committee at the Pennsylvania State University College of Medicine.

eIF4F assembly

Affinity chromatography was used to separate eIF4E containing protein complexes [6]. Frozen left lungs were pulverized using an ice-cooled, Dounce tissue homogenizer and dissolved in CHAPS lysis buffer (40 mM HEPES PH 7.5, 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM β-glycerophosphate, 40 mMNaF, 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 washed m^7-GTP Sepharose beads (GE Scientific, Piscataway, NJ) and incubated for 2 hours at 4°C. Following incubation, beads were pelleted, washed, and the protein complexes removed by boiling. Affinity purified protein complexes were separated by electrophoresis and immunoblotted with antibodies to eIF4E, eIF4G, and 4E-BP1. Immunoblots were visualized *via* chemiluminescence and quantified using a Gene Gnome Bio-Imaging System (Syngene Incorporated, Fredericksburg, MD) with blots of eIF4E, eIF4G, and 4E-BP1 developed under identical conditions within each litter set (RA and Ox). For immunoblot figures, the large number of animals and conditions necessitated "pasting" together lanes from the same gel [6].

Immunoblotting

Equal amounts of protein in CHAPS lysis buffer were electrophoretically separated on 10% Bis-Tris gels (Invitrogen/Biosource, Carlsbad, CA) and transferred to PVDF membranes [6]. Membranes were incubated with following antibodies: S6K1 (Thr^{389}), S6rp ($Thr^{235/236}$), 4E-BP1 (Thr^{70}), eIF4E, eIF4G, eIF2B, and eIF2α (all 1:1000, except S6Rp ($Thr^{235/236}$) at 1:2000; Cell Signaling Technologies, Beverly, MA); S6K1 and 4E-BP1 (1:2500 and 1:10,000, respectively, Bethyl Laboratories, Montgomery, TX); eIF2α (Ser^{51}) and eIF2Bε (Ser^{535}) (1:1000; Invitrogen); β-actin (1:5000; Sigma Chemical, St. Louis, MO); and species-specific horseradish peroxidase-conjugated secondary antibodies (1:5000; GE Scientific). Protein expression was normalized to β-actin on each gel and the ratio of phosphorylated to total proteins calculated.

Polysome analysis

Sucrose gradient centrifugation was employed to analyze lung polysome aggregation as previously described [6]. Briefly, lung tissue was homogenized in resuspension buffer [50 mM HEPES, pH 7.4; 75 mM KCl; 5 mM Mg_2Cl_2; 250 mM sucrose; 100 µg/ml cyclohexamide; 2 mM DTT; 1% Triton X-100; 1.3% deoxycholate; 10 µl/ml Super-asin (Ambion, Austin, TX)], cleared by centrifugation, and the supernatants layered onto 20-47% linear sucrose gradients. Gradients were separated at 90,000 x *g* for 4 hours at 4°C in an ultracentrifuge. Each gradient was then upwardly displaced using a Fluorinet fractionator and the optical density at 254 nm recorded on a chart recorder.

Statistical analysis

Exposures were conducted on a minimum of 2 liters per atmosphere, with the exception of the rapamycin experiment which represents a single trial using two litters. Normally distributed data were analyzed using *t*-tests or analysis of variance (ANOVA, one- or two-way) with Tukey testing to identify individual differences when appropriate. Non-normally distributed data were analyzed using Mann-Whitney or Kruskal-Wallis ANOVA on ranks with testing for multiple comparisons by Dunn's method when appropriate. For simplicity, data in figures is listed as mean ± SEM regardless of testing method and the level of significance set at $p<0.05$.

Results

Fasting suppresses mTOR activity in the lungs RA and Ox-exposed rat pups

To delineate the effect of hyperoxia on eIF4F assembly, we used maternal separation and newborn fasting followed by re-feeding with the aim of first suppressing, then stimulating, mTOR activity and eIF4F assembly in the presence of hyperoxia. Using S6K1 phosphorylation as marker of mTOR activation, preliminary studies revealed that 8 hours of fasting was necessary to reduce S6K1 phosphorylation on Thr^{389} (not shown). Even

with this period of fasting, mTOR activity was not completely suppressed, as intraperitoneal injection of rapamycin further suppressed S6K1 and 4E-BP1 phosphorylation below that of fasting levels (Figure 1). During the 8-hour fast, serum glucose levels remained unchanged independent of whether the pups were reared in RA or Ox (RA: non-fasted, 92 ± 5; fasted 105 ± 15; Ox: non-fasted, 126 ± 23; fasted 95 ± 6 mg/dl, NS). In agreement with our previous findings, rat pups exposed to 24 hours of 95% O_2 gained a similar amount of weight to those reared in room air (RA: time 0-11.2 ± 0.3 g, 24 hrs-13.2 ± 0.4 g; Ox: time 0-11.3 ± 0.2 g, 24 hrs-13.1 ± 0.2 g, NS) [6]. Weights were unaltered over the 8-hour period of fasting in either RA or Ox animals.

As illustrated in figure 2, 8 hours of fasting (Dam-fed *vs*. Water-fed) reduced the phosphorylation of S6K1 on Thr^{389} in lung tissue from both RA and Ox exposed pups, but had no effect on the phosphorylation of 4E-BP1 at Thr^{70}. Fasting also reduced the phosphorylation of the S6K1 substrate, S6Rp, though only in the RA animals (Figure 2). Formula feeding, however, failed to stimulate S6K1, S6Rp, or 4E-BP1 phosphorylation above fasted levels after 30 or 60 minutes (Figure 2). These studies suggest that while fasting is capable of suppressing mTOR activity in the lung, formula feeding-induced stimulation in the lung is rather insensitive.

Protein synthetic rates generally directly correlate with formation of active eIF4F complexes. The association of eIF4G with eIF4E (eIF4G:eIF4E) represents a surrogate for the assembly of active eIF4F complexes. Affinity purification of eIF4E from lung homogenates permits the identification of the relative association of eIF4E with both eIF4G and 4E-BP1. Using this technique, we found that fasting and re-feeding significantly altered eIF4G:eIF4E in RA and Ox animals ($p<0.05$) (Figure 3). In RA-exposed pups, eIFG:eIF4E doubled 60 minutes post-feeding (Water-fed: 0.91 ± 0.22 *vs*. 60 min-fed: 1.88 ± 0.2, $p<0.05$-*t* test). By comparison, in Ox animals maximal eIF4G:eIF4E occurred 30 minutes post-feeding (Water-fed: 1.00 ± 0.20 *vs*. 30 min-fed: 1.99 ± 0.21, NS – Mann-Whitney). Availability of eIF4E is regulated by the release from 4E-BPs, the phosphorylation of which is mTOR-dependent. Assessing of the association of 4E-BP1:eIF4E in the same samples revealed that fasting increased 4E-BP1 binding in both RA and Ox-exposed animals (RA: Dam-fed, 1.0 ± 0.0 *vs*. Water-fed, 2.6 ± 0.5; Ox: Dam-fed, 1.2 ± 0.1 vs. Water-fed, 3.2 ± 0.6, both $p<0.001$). Neither feeding nor hyperoxia, however, altered 4E-BP1:eIF4E.

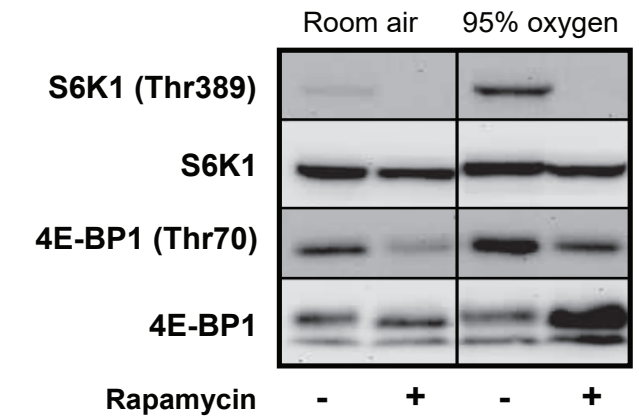

Figure 1: Fasting fails to completely suppress pulmonary mTOR activity. Rat pups received i.p. injection of rapamycin (4 mg/kg) or an equivalent volume of DMSO prior to exposure to room air or 95% O_2 for 24 hrs. After 24 hrs, pups were fasted for 8 hrs by maternal separation, gavage fed sterile H_2O, and lungs collected 30 min later. Figure depicts representative immunoblots of the phosphorylation of downstream mTOR substrates, S6K1 and 4E-BP1 (n=3 pups/condition)

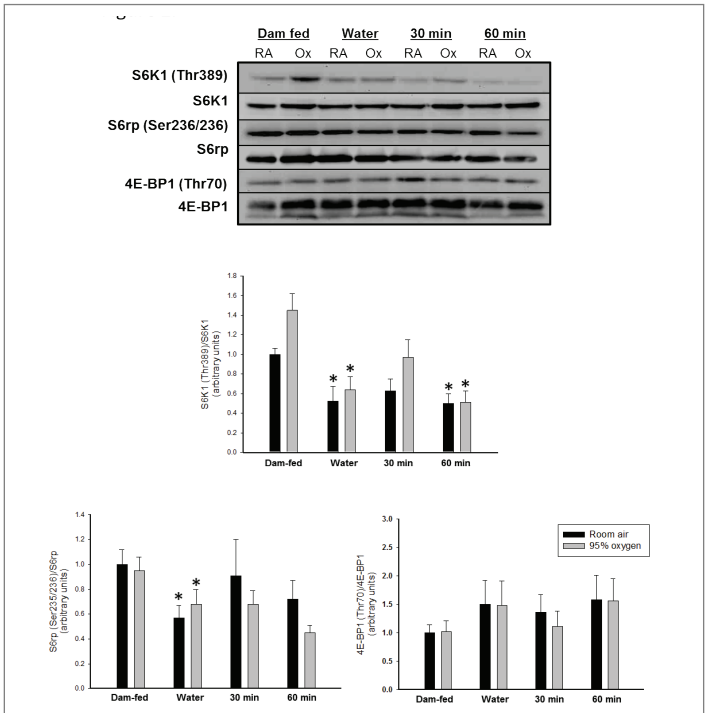

Figure 2: Feeding fails to stimulate mTOR activity in RA and Ox-exposed rat pups. A) Immunoblots are representative of thephosphorylation of S6K1, S6Rp, and 4E-BP1. Histograms (B-D) show the mean phosphorylation in the lungs of room air (black) and 95% O_2-exposed (gray) pups. Columns represent mean (n=11-12) and bars SEM. "*" denotes a significant difference ($p<0.05$) compared to dam-fed pups.

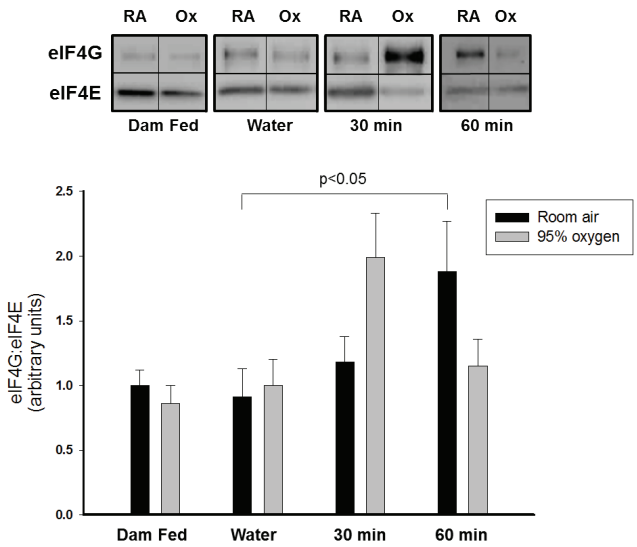

Figure 3: Hyperoxia does not suppress meal-stimulated eIF4F assembly. Affinity purified lung extracts were separated by electrophoresis and the relative amount of eIF4G co-precipitating with eIF4E identified by immunoblotting. A) Representative immunoblots depict a single animal in each group. Lines of separation represent non-adjacent lanes. B) Histograms display relative association of eIF4G with eIF4E. Columns (black – room air; gray – 95% O_2) represent means (n=11-12) and bars SEM. Analysis found a positive effect of feeding on eIF4G:eIF4E binding in RA pups (ANOVA, $p=0.038$) with a difference identified between water- and formula-fed pups at 60 min ($p<0.05$). For Ox pups, testing revealed an effect of feeding (Kruskal-Wallis, $p = 0.041$), but no individual differences.

The relative insensitivity of the lung to meal-feeding raised concern a cow's milk-based formula may not stimulate translational regulatory pathways in the newborn rat. As a control, we studied meal-stimulated eIF4F assembly in skeletal muscle and liver. Figure 4 demonstrates that formula feeding following an 8-hour fast dramatically stimulates active eIF4F complex assembly in liver but has little effect in gastrocnemius muscle. The combined results from this series of experiments indicate that cow's-milk feedings can increase translational activity in a tissue-specific manner and that hyperoxia does not blunt this response in the lung.

Hyperoxia suppresses polysome aggregation irrespective of feeding

An increase in the polysomal to non-polysomal RNA ratio is indicative of a relative enhancement of initiation events. Figure 5 illustrates that polysome aggregation was not responsive to formula feeding in either group, a finding consistent with the lack of change in S6K1 and 4E-BP1 phosphorylation. Hyperoxia, however, decreased the ratio of polysomal to non-polysomal RNA in response to both water and formula feeding (2-way ANOVA, $p<0.05$). Interpretation of this finding suggests non-eIF4F events inhibit mRNA translation at the level of initiation.

Hyperoxia induces sustained phosphorylation of eIF2α

Similar to our previous observations, we found that hyperoxia tends to increase eIF2α phosphorylation on Ser^{51} during continuous feeding (RA: 1.0 ± 0.1 *vs.* Ox: 3.5 ± 1.0, p=0.08, figure 6) [6]. In comparison to water, formula feeding neither increased nor decreased eIF2α phosphorylation in either atmospheric group. Among the formula fed animals, the phosphorylation of eIF2α was greater 60 minutes post-feeding in Ox- compared to RA-exposed pups (RA: 1.2 ± 0.3 *vs.* Ox: 2.9 ± 1.0, $p<0.05$, figure 6). Analysis of eIF2Bε, on the other hand, failed to identify an effect of feeding on eIF2Bε expression or phosphorylation on Ser^{535} (2-way ANOVA).

Discussion

Aggressive nutritional support of premature infants at risk for the development of BPD is standard practice in neonatal intensive care. Despite these efforts, nutritional deficiencies persist in this population illustrated by the high incidence of growth failure relative to gestational

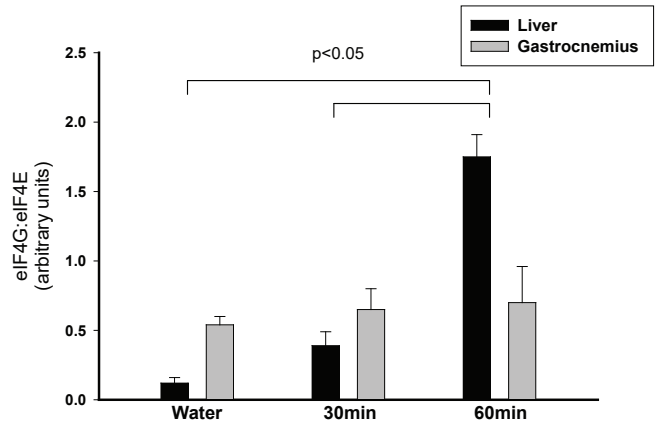

Figure 4: Feeding induces eIF4F assembly in liver. Affinity-purified liver and gastrocnemius extracts were separated by electrophoresis and the amount of eIF4G co-precipitating with eIF4E determined. Panel A shows representative immunoblots from a single animal for each tissue. B) Histogram shows the relative binding of eIF4G with eIF4E. Columns represent means ± SEM (n=3) and bars SEM. Statistical analysis revealed that 60 min after feeding eIF4G:eIF4E binding increased relative to water-fed or meal-fed pups at 30 min ($p<0.05$, *t* test)

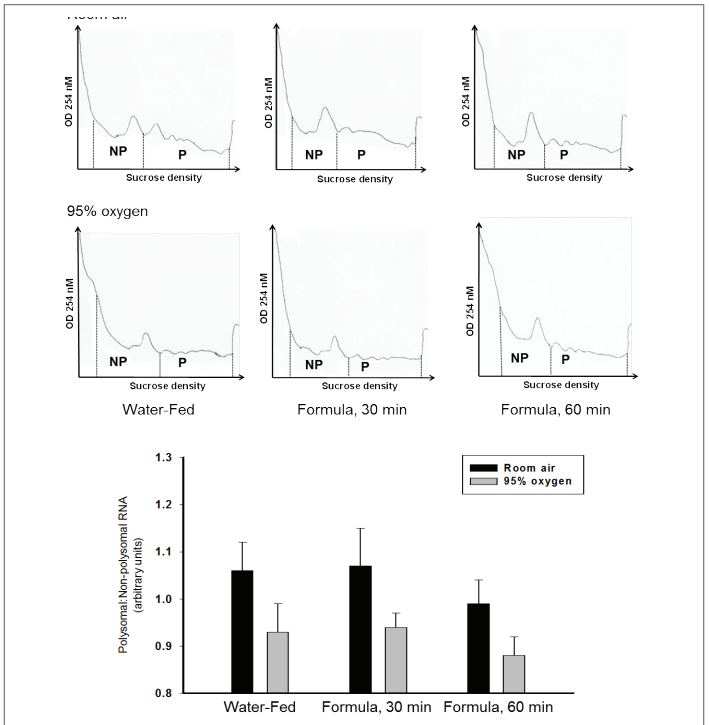

Figure 5: Hyperoxia suppresses polysome aggregation irrespective of feeding. Total lung RNA from pups exposed to RA or Ox was subjected to sucrose density gradient centrifugation as described under Methods. A) Tracings represent the quantity of RNA at specific sucrose density as measured by OD 254 nm. The dashed lines separate non-polysomal (NP) and polysomal (P) RNA. B) The mean ratio of P/NP for RA (black) and Ox (gray) animals is represented by columns (n=6 per feeding condition/atmosphere). Bars represent SEM. Two-way ANOVA revealed a significant effect of Ox on polysome aggregation (p=0.012), but no feeding effect in RA or Ox animals

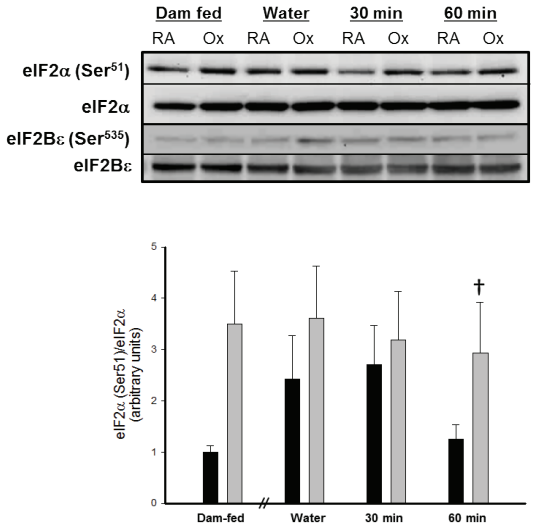

Figure 6: Effect of 95% O_2 on phosphorylation of eIF2α.A) Immunoblots represent the relative phosphorylation of eIF2α and eIF2Bε. B) Histograms show the mean phosphorylation of eIF2α in RA (black) and Ox (gray) pups. Columns represent mean (n=12) and bars SEM. In dam-fed animals, eIF2α phosphorylation tended to be higher in Ox than in RA pups (p=0.08). Formula-feeding failed to alter eIF2α phosphorylation in animals reared in either atmosphere. "†" denotes a significant difference ($p<0.05$) between RA and OX at 60 min.

age-matched controls [16]. Animal studies provide convincing evidence for a global impact of under-nutrition, encompassing reductions in lung weight, alveolar number, elastin and hydroxyproline content, and type II cell maturation [17-19]. Growth failure in preterm infants is further confounded by risk factors that contribute to the development of BPD [20,21]. In newborn guinea pigs, restriction of nutrient intake enhances the toxicity of hyperoxia, leading to increased mortality without altering pulmonary inflammation or antioxidant capacity [22]. Whether hyperoxia alters nutritional signaling regulating mRNA translation in the lung and whether such changes influence lung growth remains to be established. Exposure to95% O_2 attenuates pulmonary protein synthesis and transiently augments the activity of mTOR in continually nursing term, newborn rats [6]. The current study expands upon these earlier findings by illustrating that hyperoxia does not alter feeding-induced initiation complex assembly or major signaling events regulating translation initiation and by reaffirming that hyperoxia-induced reductions in polysome aggregation correlate with eIF2α phosphorylation.

Within the lung, colostrum, mature porcine milk, and casein-based formula all increase the FSR following fasting, while only colostrum enhances protein synthetic capacity (RNA/protein ratio) [13]. The stimulatory effect of feeding in the lung is responsive to changes in serum amino acid levels, not insulin levels, denoting nutritional protein as a major contributor to the growth [23]. Based upon methodology designed to investigate meal-stimulated effects in skeletal muscle from newborn pigs, the present study examined the impact of hyperoxia on nutritional signaling at 30 and 60 minutes post-feeding [14]. Although an 8-hour fast was sufficient to reduce S6K1 and S6Rp phosphorylation and increase 4E-BP1:eIF4E association, it did not produce the anticipated reductions in 4E-BP1 phosphorylation or eIF4G:eIF4E assembly in either the RA or Ox exposed animals. The disparate response to fasting may be explained by the insensitivity of Thr^{70} to fasting relative to other phosphorylation sites or the presence of fasting resistance eIF4F factors. Differences in the molecular weights of fetal and adult eIF4G1 and in eIF4A1/2 expression have been proposed to explain fetal hepatic rapamycin resistance, an effect independent of mTOR substrate phosphorylation [24]. Although the current study was not designed to detect such changes, the results are entirely compatible with a developmental paradigm which fosters eIF4F assembly even in the presence of hyperoxia.

Activation of translational signaling components by feeding has been extensively investigated in skeletal muscle and liver of newborn piglets. In skeletal muscle, dietary protein stimulates Akt, S6K1, and 4E-BP1 phosphorylation and the assembly of active eIF4F complexes [25]. In stark contrast, complete meal-feeding in the present study did not enhance the phosphorylation of S6K1, S6Rp, or 4E-BP1, but still stimulated the formation of eIF4G:eIF4E complexes in both RA and Ox treated pups. Phosphorylation of eIF4G on Ser^{1108} is directly correlated with active eIF4G:eIF4E assembly in several studies, including investigations of feeding-induced protein synthesis in the skeletal muscle [26]. Rapamycin-independent eIF4G phosphorylation has been linked to PKCε activation following feeding raising the possibility that mTOR-independent eIF4F formation might also be involved in the lung [26].

Phosphorylation of eIF2α increases the affinity of eIF2-GDP for eIF2B 150-fold, indicating that relatively minor increases in phosphorylation markedly inhibit cap-dependent mRNA translation [27]. In RA exposed newborn rats, fasting and re-feeding gradually, though not significantly, enhance eIF2α phosphorylation until 30 minutes at which point phosphorylation declines toward the dam-fed state. This temporal pattern of eIF2α phosphorylation is consistent with activation of GCN2 (General Control Non-derepressible-2), the eIF2α kinase responsive to amino acid deprivation [28]. While the phosphorylation of eIF2α tends to be greater in hyperoxia, the effect does not reach statistical significance until 60 minutes post-prandial. Within the Ox group, eIF2α phosphorylation parallels polysome aggregation more closely than eIF4F assembly, suggesting that eIF2α, alone or in tandem with other signaling events regulating initiation, inhibits feeding-stimulated initiation events. The lack of association between polysome aggregation and eIF4F assembly in RA pups may reflect equal changes in initiation and elongation rates during fasting, resulting in a consistently higher polysomal:non-polysomal RNA (P/NP) ratio.

Although the findings presented herein suggest that hyperoxia has little effect on the mTOR signaling pathways regulating initiation, some concerns remain. The use of a human whey/casein-based formula may have been insufficient to stimulate mTOR signaling. Mature rat milk or a casein formula based upon the composition of rat rather than human milk may have produced different results. Ideally, assessment of feeding induced translational effects would also include measuring protein synthetic rates. This approach was consciously omitted to avoid introduction of an additional stressor in the immediate post-prandial period and to avoid prolonged removal from hyperoxia. Even without this potentially useful data, the selected methodology was capable of stimulating eIF4F assembly and discerning differences between RA and Ox animals.

In summary, the results from this study show that hyperoxia reduces the ability of feeding to globally stimulate translation initiation in the lung possibly through mechanisms involving stress-induced phosphorylation of eIF2α. Given that whole body protein synthetic rates in mammals are higher in the immediate postnatal period than during any subsequent phase of life, brief reductions in polysomal aggregation have the potential to alter the expression of growth factors integral to critical developmental windows [29]. The rapid alveolarization following birth renders the lung uniquely vulnerable to the negative influence of hyperoxia on protein synthesis, with the potential to adversely influence life-long lung function

Statement of Financial Support

This work was funded in part by a grant from the Children's Miracle Network at the Penn State Children's Hospital.

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