Hyperoxia-induced activation of the integrated stress response in the newborn rat lung

Wesley M. Konsavage,1 Lianqin Zhang,1 Yuchieh Wu,1 and Jeffrey S. Shenberger1,2,3

1Departments of Pediatrics and 2Cellular and Molecular Physiology and 3CHILD Research, The Pennsylvania State College of Medicine, Hershey, Pennsylvania

Submitted 3 June 2011; accepted in final form 4 October 2011

Konsavage WM, Zhang L, Wu Y, Shenberger JS. Hyperoxia-induced activation of the integrated stress response in the newborn rat lung. Am J Physiol Lung Cell Mol Physiol 302: L27–L35, 2012. First published October 7, 2011; doi:10.1152/ajplung.00174.2011.—Diverse environmental stresses stimulate eukaryotic translation initiation factor 2α (eIF2α) phosphorylation, leading to a stress-resistant state characterized by global attenuation of protein synthesis and induction of cytoprotective genes. The signal transduction network culminating in these effects is referred to as the integrated stress response (ISR) or, when initiated by misfolded proteins within the endoplasmic reticulum (ER), the unfolded protein response (UPR). Given that we previously reported that exposure of 4-day-old Sprague-Dawley rats to 95% O2 (Ox) diminishes global pulmonary protein synthesis and increases eIF2α phosphorylation, we conducted the current study to determine whether Ox activates the ISR or UPR. We found that Ox-induced alterations in ER morphology of alveolar type II cells and interstitial fibroblasts were not associated with activation of the UPR sensors PERK or activating transcription factor (ATF) 6 or with X-box binding protein-1 mRNA splicing in whole lung extracts. Exposure to Ox enhanced ATF4 immunoreactivity and nuclear protein content, followed by a 2- and 5-fold increase in ATF3 protein and mRNA expression, respectively. The accumulation of nuclear ATF4 coincided with induction of glutamate-cysteine ligase catalytic subunit, an ISR-responsive gene. Immunohistochemistry revealed that changes in ATF3/4 expression were prominent in the alveolus, whereas primary cell culture implicated epithelial and endothelial cells as targets. Finally, induction of ISR intermediates in the intact lung occurred in the absence of the phosphorylation of PKR, JNK, ERK1/2, and p38 MAPK. These findings demonstrate that Ox activates the ISR within the newborn lung and highlight regional and cell-specific alterations in the expression ISR transcription factors that regulate redox balance.

unfolded protein response; eukaryotic initiation factors; lung injury; bronchopulmonary dysplasia

HIGH INSPIRED O2 CONCENTRATIONS used in the treatment of surfactant-deficient preterm infants contribute to the pathogenesis of bronchopulmonary dysplasia (BPD), a leading cause of pediatric chronic lung disease. Exposure to hyperoxia generates reactive species that interfere with vital cellular processes integral to the survival and growth of lung epithelial cells, a primary cellular target in BPD. Cellular adaptation to environmental stress frequently involves the phosphorylation of the α-subunit of eukaryotic translation initiation factor 2 (eIF2α) on Ser51, which inhibits global mRNA translation by preventing the recruitment of the initiator Met-tRNA^Met to the 40S ribosome (2). Diverse environmental stresses, including oxidants, UV irradiation, and amino acid depletion converge at eIF2α to activate a set of common cytoprotective genes, collectively known as the integrated stress response (ISR) (17, 20). Although our laboratory recently reported that hyperoxia attenuates global protein synthesis coincident with a reduction in higher order polysomes and increases phosphorylation of eIF2α in the lungs of newborn rats, it is unclear whether hyperoxia activates the ISR signaling under these conditions (22).

Inhibition of cap-dependent mRNA translation by phosphorylated eIF2α promotes the cap-independent translation of activating transcription factor (ATF) 4 via upstream open reading frames (42). Increased ATF4 expression activates stress-responsive transcriptional factors including ATF3, a member of the basic-region leucine zipper (bZIP) family of transcription factors and the cyclic AMP response element-binding (CREB) subfamily (20). Within the ISR program, ATF3 induction influences multiple biological processes including development, apoptosis, antioxidant responses, DNA damage, and immune modulation (11, 13, 17, 40). In a mouse model of ventilator-induced lung injury, loss of ATF3 upregulates the expression of genes containing putative ATF3 cis-regulatory sequences: IL-6, IL-12b, CCL4, fibroactin, urokinase receptor (Plau-R), Myc, and IFN-γ. Ventilated ATF3 null mice show increased cell infiltration in the lung and bronchoalveolar lavage, increased pulmonary edema, and indexes of tissue injury implicating ATF3 as a cytoprotective and anti-inflammatory transcriptional modulator (1).

Phosphorylation of eIF2α by any of the four known stress-responsive kinases [heme-regulated inhibitor (HRI), general control non-derepressible-2 (GCN2), double-stranded RNA-dependent protein kinase (PKR), and PKR-like endoplasmic reticulum kinase (PERK)] leads to ISR activation (14, 44). Although multiple stressors stimulate these kinases, stress generated by the endoplasmic reticulum (ER) via PERK is unique. Within the ER, chaperones bind unfolded proteins to “shield” them from protein-protein interactions and to promote the completion of folding. During certain physiological or pathological conditions, the client load of incompletely folded proteins may exceed chaperone levels generating “ER stress,” which, if left unchecked, collapses the cellular secretory capacity and triggers apoptosis. As protection, cells have developed a complex homeostatic mechanism known as the unfolded protein response (UPR), which represses mRNA translation to reduce the protein load entering the ER, augments folding capacity by increasing ER chaperone expression, and enhances ER-associated degradation of protein aggregates (29).

The UPR involves activation of three transmembrane sensors, inositol-requiring protein 1 (IRE1), ATF6, and PERK, which are tethered to the ER chaperone binding immunoglobulin protein (BiP) (16). Misfolded proteins “attract” BiP, acti-
vating the stress sensors and secondary transcription factors [X-box binding protein-1 spliced (XBP1s), ATF6, and ATF4] that enhance the expression of genes necessary to restore ER homeostasis (3, 20). Because signaling downstream of ATF4 is common to the ISR, the UPR essentially represents an “expanded” ISR signaling program specifically responsive to ER stress (see Fig. 1). Although recent evidence indicates that hyperoxia does not activate the UPR in lung epithelial cell lines and adult mice despite reducing BiP protein expression, it is unclear whether the hyperoxia-induced eIF2α phosphorylation evident in newborn rat lungs represents activation of the ISR alone (10, 25).

Given the potential protective benefits of the ISR, understanding the nature and extent of ISR/UPR activation in the hyperoxia-exposed lung is crucial to untangling the mechanisms leading to the arrest of alveolar development observed in BPD and to designing treatment modalities that preserve lung architecture and function. The objective of the present study, therefore, is to identify the capacity for hyperoxia to activate the ISR within the lungs of newborn rats. On the basis of our previous work, we hypothesized that hyperoxia would activate ISR, but not UPR, signaling within alveolar cells.

METHODS

Animal model and conditions. Animal exposures were conducted in a manner identical to that previously described (22). Briefly, timed-pregnant Sprague-Dawley rats (Charles River Laboratories, Boston, MA) in standard rat cages were housed in Plexiglas exposure chambers and exposed to 72 h of room air (RA) or 95% O₂ (Ox) beginning on day-of-life (DOL) 4 and ending on DOL7. Delivery of 100% O₂ was continually adjusted using a computerized system (OxyCycler A; BioSpherix, Redfield, NY) to ensure a constant atmosphere of 95% O₂. The concentration of CO₂ measured by the BioSpherix sensor was maintained <0.5%. Dams were interchanged daily between groups to minimize O₂ toxicity. Animals were supplied with rat chow and water ad libitum, exposed to 12:12-h day-light cycles, and maintained at 26°C and 75–80% humidity. All animal protocols were approved by the Institutional Animal Care and Use Committee at the Pennsylvania State University College of Medicine.

Immunoblotting. Whole left lung tissue was homogenized in 3-(3-chloro-1H-indol-2-yl)(2-methylammonio)-1-propanesulfonate (CHAPS) lysis buffer using the Bullet Blender (NextAdvance, New Averill, NY). Equal amounts of protein were electrophoretically separated on 10% Bis-Tris gels (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride membranes as previously described (22). Membranes were incubated with the following antibodies: PERK, BiP, eIF2α, extracellular-regulated kinases 1/2 (ERK1/2), ERK1/2 (Tyr202/Tyr204), e-Jun NH2-terminal kinase (JNK), JNK (Thr183/Tyr185), Hsp40, Hsp90, Grp94, GCN2, GCN2 (Thr898), protein disulfide isomerase (PDI), and e-Jun (1:1,000; Cell Signaling Technologies, Beverly, MA); eIF2α (Ser51) and p38 MAPK (Thr180/Tyr182) (1:1,000; Invitrogen); β-actin (1:5,000; Sigma Chemical, St. Louis, MO); Hsp70 (1:5,000; Enzo Life Sciences, Plymouth Meeting, PA); ATF3, ATF4, ATF6, PKR, PKR (Thr446), and p38 MAPK (all 1:500, except p38 1:1,000; Santa Cruz Biotechnologies, Santa Cruz, CA) and species-specific horseradish peroxidase-conjugated secondary antibodies (1:5,000; GE Scientific, Piscataway, NJ). Protein expression was normalized to β-actin.

XBP1 mRNA splicing. Total RNA was isolated from individual right upper lobes of RA- or Ox-exposed animals using the RNeasy mini kit (Qiagen, Valencia, CA). XBP1 mRNA was amplified with the One-Step RT-PCR kit (Qiagen) using 0.5 μg of the isolated RNA. PCR was carried out under the following conditions: 50°C for 30 min, 95°C for 15 min, 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and 72°C for 10 min. The following primers were used: XBP1 forward, ACGAGGAAAAACTCTAGGC; and XBP1 reverse, ACAGGGTCACAATGGTCAGC (41). Products were separated by electrophoresis on a 2% agarose gel. Rat2 fibroblast cells (CRL-1764; ATCC, Manassas, VA) treated with 4 mM thapsigargin were used as a species-identical positive control study to illustrate splicing of XBP1.

Quantitative real-time PCR. Total RNA was isolated as described above and reverse transcribed to cDNA using the High Capacity cDNA reverse transcription kit (Life Technologies/ABI, Carlsbad, CA). Reaction conditions were: 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. Quantitative RT-PCR was performed on a 1:8 dilution of the generated cDNA using the Quantitect SYBR green PCR kit (Qiagen). Reaction conditions were 50°C for 2 min, 95°C for 15 min, and 45 cycles of 95°C for 20 s, 55°C for 30 s, and 72°C for 1 min. The following primers were used: XBP1 forward, ACGAGGAAAAACTCTAGGC; and XBP1 reverse, ACAGGGTCACAATGGTCAGC (41). Products were separated by electrophoresis on a 2% agarose gel. Rat2 fibroblast cells (CRL-1764; ATCC, Manassas, VA) treated with 4 mM thapsigargin were used as a species-identical positive control study to illustrate splicing of XBP1.
the specificity of the amplified PCR product was verified by the analysis of the melting curves, which were product specific.

Cytoplasmic/nuclear fractionation. Cytoplasmic/nuclear fractionation was performed as described by Ertsey et al. (9). Lung tissue was powdered in liquid N₂ using a mortar and pestle, followed by Dounce homogenization on ice using 4 volumes of cytoplasmic extraction buffer [10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM MgCl₂, 0.2% NP-40, 1 mM DTT, and protease inhibitors (Roche)]. Nuclei were isolated by centrifugation at 720 g for 5 min at 4°C, and the supernatant was collected and saved as the cytoplasmic fraction. The nuclei were then washed three times in cold PBS and resuspended in 2 X original volumes of nuclear lysis buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM MgCl₂, 25% glycerol, 1 mM DTT, and protease inhibitors). Nuclei were incubated on ice for 10 min with occasional vortexing, followed by centrifugation at 16,000 g for 10 min at 4°C. The supernatant was collected and saved as the nuclear fraction. Equal amounts of nuclear protein were separated by electrophoresis, and the amounts of ATF3 and ATF4 were compared at each time point. Purity of fractions was tested using sera, sections were incubated overnight at 4°C. The nuclei were then washed three times in cold PBS and resuspended in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. The tissue was fixed, dehydrated through graded ethanol and water. The sections were subjected to antigen retrieval by boiling in 10 mM sodium citrate buffer (pH 6.0), and endogenous peroxidase activity was quenched by incubation in 10% neutral buffered formalin, and the left lung was embedded in optimum cutting temperature compound, and cut into 1-mm³ pieces and continually fixed for 24 h. After being washed with 0.1 M sodium cacodylate buffer, the tissue was postfixed overnight in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. The tissue was then rinsed in 0.1 M sodium cacodylate buffer, dehydrated in a graded series of ethanol, incubated with propylene oxide/epoxy resin mixture (50:50), and embedded in Epon 812 (Electron Microscopy Sciences, Hatfield, PA). Sections 70–90 nm in thickness were cut, mounted on 200-mesh copper grids, and stained with aqueous uranyl acetate and lead citrate. Sections were examined using a JEOL JEM-1400 transmission electron microscope (Tokyo, Japan).

Immunohistochemistry. Lungs were inflated fixed at 25 cmH₂O with 10% neutral buffered formalin, and the left lung was embedded in paraffin. Paraaffin sections (10 μm) were deparaffinized with xylene and rehydrated through graded ethanol and water. The sections were subjected to antigen retrieval by boiling in 10 mM sodium citrate buffer (pH 6.0), and endogenous peroxidase activity was quenched by incubation in 3% H₂O₂ for 10 min. After being blocked in 5% normal goat serum, sections were incubated over night at 4°C in rabbit ATF3 and ATF4 antibodies (both 1:50; Santa Cruz). Sections were then incubated with biotinylated goat anti-rabbit IgG and stained with the Vectastain ABC Elite kit (both Vector Laboratories, Burlingame, CA) using 3,3'-diaminobenzidine (Sigma-Aldrich) as the chromagen. Control sections were treated identically except that the same species of IgG replaced the primary antibody. Counterstaining was carried out using hematoxylin.

Immunofluorescence microscopy. Lungs were frozen in liquid N₂, embedded in optimum cutting temperature compound, and cut into 6-μm sections. Sections were double labeled with the mixture of rabbit anti-eIF2α (1:50, Ser51; Abcam, Cambridge, MA) and mouse anti-vimentin (1:50; Abcam) in 0.25% BSA-PBS overnight. After being incubated in PBS, sections were incubated with the mixture of mouse anti-rabbit Cy3-labeled (1:200), donkey anti-rat IgG and donkey anti-mouse DyLight 488 labeled (1:200) secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) in 0.25% BSA-PBS for 1.5 h at room temperature. The sections were viewed using an Olympus fluorescence microscope.

Cell culture. Control studies illustrating UPR activation in rat cells were performed on the Rat2 cells treated with 1–4 μM thapsigargin dissolved in DMSO. Rat2 cells were grown in DMEM containing 100 IU/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine, and 10% FBS. Equal quantities of cell lysate were resolved by immunoblotting as described above.

The ability of hyperoxia to activate the ISR in individual cell types was assessed by exposing neonatal human lung fibroblasts (HLF), human pulmonary microvascular endothelial cells (PMEC; Promocell, Heidelberg, Germany), and human small airway epithelial cells (HSAEC; Lifeline Cell Technologies, Frederick, MD) to 6–24 h of 95% O₂ using a BioSpherix OxyCycler C designed for cells as previously described (22). HLF were grown in DMEM containing 100 IU/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine, and 10% FBS, whereas PMEC were grown in endothelial growth medium MV (PromoCell), and HSAEC were grown in epithelial growth medium (Lifeline). Equal quantities of cell lysate were resolved by immunoblotting and blotted for components of the ISR signaling pathways.

Statistical analysis. All exposures were conducted a minimum of three times. Normally distributed data with equal variances were tested using two-way analysis of variance (ANOVA) with Fisher’s least square difference post hoc analysis used to identify individual differences. Remaining data were analyzed using the Mann-Whitney rank sum test. Normally distributed data are expressed as means ± SE, whereas nonnormally distributed data are given as median values with the 25th and 75th percentiles. For all testing, the level of significance was set at P < 0.05.

RESULTS

Hyperoxia alters ER morphology of select lung cells. Activation of the UPR is commonly associated with alterations in ER morphology (15). As an initial exploration of the potential for hyperoxia to activate the UPR, we examined ER morphology in the lungs of newborn mice. Although it was not possible to selectively isolate each pulmonary cell type, our descriptive analysis centered on alveolar type I and II epithelial cells differentiated by the presence of lamellar bodies, interstitial fibroblasts, airway epithelial cells, and capillary endothelial cells. After examining in excess of 20 grids from multiple animals in each group, we identified little change in the ER morphology of airway epithelial, type I, or endothelial cells. As shown in Fig. 2, hyperoxia was associated with blurring of ER border margins, dilatation, and loss of ribosome adherence in type II cells and interstitial fibroblasts after 24 h. This pattern was more common in interstitial fibroblasts than in type II cells and appeared more prominent at 24 than 72 h.

Hyperoxia does not activate the UPR within the lung. The UPR involves the coordinated activation of each of three transmembrane ER stress sensors. To investigate activation of the eIF2α kinase PERK and the transcription factor ATF6, we performed immunoblotting of whole right upper lobes lysates from RA- and Ox-exposed rat pups, looking for the PERK mobility shift and the 50-kDa cleaved isoform of ATF6 indicative of the phosphorylation/activation and active, nuclear-localizing transcription factor, respectively. The representative immunoblots in Fig. 3A illustrate that Ox had no effect on PERK mobility, ATF6 cleavage, or BiP protein expression. Hyperoxia tended to increase overall PERK protein expression at 24 and 72 h and to reduce BiP protein expression at 24 h, but these did not reach statistical significance. Lung lysates from pups from individual litters examined after 8 and 16 h of hyperoxia also demonstrated no alterations in PERK phosphorylation/mobility or ATF6 cleavage (data not shown). Figure 3B provides an example of a PERK mobility shift and ATF6 cleavage in Rat2 fibroblasts treated with the UPR activator thapsigargin to confirm the ability of the antibodies to detect changes in rat cells. During UPR activation, dissociation of IRE1 from BiP induces endonuclease activity, which generates the transcription factor XBP1s (XBP1 spliced). Employing primers known to detect both XBP1u and XBP1s, we found that hyperoxia failed to induce splicing at either 24 or 72 h of hyperoxia.
exposure (Fig. 3C), whereas thapsigargin treatment of control Rat2 fibroblasts clearly generated the smaller XBP1s product (41). Finally, we examined downstream effectors of the UPR that help restore ER homeostasis, namely, resident heat shock proteins and chaperones (30). We found no changes in the protein expression of Grp94, Hsp90, Hsp70, Hsp40, and PDI at either 24 or 72 h in response to Ox (not shown). When viewed together, these studies show that hyperoxia does not activate any arm of the UPR within the whole lung.

Hyperoxia activates the ISR. Convergence of stress signals at eIF2α may also activate the ISR to conserve cellular energy by inhibiting cap-dependent mRNA translation, restore intracellular redox balance, and induce apoptosis in irreparably damaged cells (20). Suppression of protein synthesis by phosphorylated eIF2α promotes the translation of the bZIP transcription factor ATF4, which in turn induces the transcriptional upregulation of ATF3 (20). In the lungs of newborn rats, ATF4 protein expression, but not ATF3 expression, was reduced from DOL4 to DOL7, independent of the fractional concentration of inspired O2 (Fig. 4, A and B). Although Ox tended to increase ATF4 protein expression, the effect did not quite reach statistical significance (2-way ANOVA, P = 0.06). Hyperoxia did, however, induce a fivefold increase in ATF3 protein expression at 72 h (P < 0.001) and resulted in a significant interaction between time and atmosphere (Fig. 4C, P < 0.001).

As shown in Fig. 5, differences in ATF3 protein expression correlated with increases in ATF3 mRNA expression, whereas the time-dependent changes in ATF4 protein did not. Findings consistent with the transcriptional regulation of ATF3 but not ATF4 (Fig. 5, A and B). Activation of transcription factors requires DNA binding and, accordingly, nuclear localization. To assess the potential for alterations in ATF expression to signify activity, we fractionated whole lobe lysates into nuclear and cytoplasmic pools. Fractionation revealed that Ox increased the nuclear content of ATF4 protein at 24 h (RA: 1.0 ± 0.2; Ox: 1.9 ± 0.2 arbitrary units, P < 0.01) but not at 72 h. Exposure to Ox tripled ATF3 protein content at 72 h (RA: 1.0 ± 0.1; Ox: 2.9 ± 0.4 arbitrary units, P < 0.01) (Fig. 6A).

Hyperoxia increases ATF4 and ATF3 expression in alveolar cells. To localize changes in ISR expression within the lung, ATF4 and ATF3 expression was assessed using immunohistochemistry. Examination of multiple sections from several pups revealed that Ox increased the expression of ATF4 at 24 h and of ATF3 at 72 h at the alveolar level (Fig. 6B). Within large conducting airways, little change in ATF4 or ATF3 expression was noted. Previously, our laboratory reported that hyperoxia increases eIF2α phosphorylation in cells within the airway and parenchyma (22). To further refine those findings, we performed dual-labeling immunofluorescent microscopy using eIF2α (Ser51) and vimentin antibodies. Using this technique, we found that the majority of cells staining positively for phosphorylated eIF2α (orange) were negative for vimentin (green), suggesting that they are less likely to be of mesenchymal origin (Fig. 6C, example depicted at 72 h). Although eIF2α (Ser51) staining was present in both RA and Ox sections, staining was clearly greater in Ox tissues.

Because we were unable to perform dual labeling with eIF2α (Ser51) and ATF4 or ATF3 antibodies, we chose to investigate the ability of hyperoxia to activate the ISR in primary lung cells. We selected human cells to illustrate the relevance of our findings to the investigation of oxygen toxicity in humans. As shown in the representative immunoblots in Fig. 7, Ox increased eIF2α phosphorylation and the expression of ATF3 and ATF4 in HSAEC and PMEC, but not in HLF. The eIF2α response in HSAEC diminished with successive passages, illustrating that time in culture influences ISR activation in certain cells. Hyperoxia also increased ATF3 protein expres-
sion in HLF without increasing eIF2α phosphorylation, indicating non-ISR signaling events are involved in distinct cells.

**ISR activation is not correlated with changes in MAPK or PKR.** Although Ox-induced eIF2α phosphorylation and the subsequent increase in ATF3 protein/mRNA expression are consistent with ISR activation, additional signaling pathways are known to regulate ATF3 expression as suggested by the cell culture data. Each of the MAPKs (JNK, p38 MAPK, and ERK1/2) induce ATF3 expression in response to various stimuli (19, 26). Evaluation of the MAPK activation in lung lysates of hyperoxia-exposed pups revealed that Ox did not induce the phosphorylation of JNK, p38 MAPK, or ERK1/2 after 24 or 72 h of exposure (not shown).

As defined, activation of the ISR is mediated by any of the eIF2α kinases. Examination of the kinase responses in primary cells did not detect Ox-mediated alterations in PKR (Thr446) or GCN2 (Thr809) phosphorylation in either HSAEC or PMEC, phosphorylation changes often associated with enhanced kinase activity (not shown). Immunobots of rat pup lung lysates also failed to identify alterations in PKR phosphorylation at Thr446 in response to hyperoxia, which was recently observed in adult mouse lungs (Fig. 8A) (25). The lack of available HRI and GCN2 phosphorylation-specific antibodies that reliably

---

**Fig. 3.** Hyperoxia does not activate the UPR in newborn rat lungs. **A**: representative immunoblots of whole lung lysates from rat pups exposed to 24 and 72 h of RA and Ox. ATF6 represents the 90-kDa uncleaved isoform (inactive) and the cleaved 50-kDa isoform (active). Ox tended to increase PERK expression without inducing a band shift indicative of phosphorylation. **B**: immunoblot illustrating expected PERK band shift and ATF6 cleavage patterns indicative of UPR activation in Rat2 cells treated with thapsigargin (4 mM) at 4–24 h. Black lines within the immunoblots represent noncontiguous portions of the same gel or a different gel using identical loading and samples. **C**: representative agarose gel electrophoresis of XBP1 PCR products illustrating both the XBP1s (spliced) and XBP1u (unspliced) isoforms (last 4 lanes). Rat2 cells treated with thapsigargin (0–4 mM) were used as control (first 3 lanes).

---

**Fig. 4.** Ability of hyperoxia to activate the ISR. **A**: representative immunoblots of whole lung lysates from rat pups exposed to 24 and 72 h of RA and Ox. Black lines within the immunoblots represent noncontiguous portions of the same gel or a different gel using identical loading and samples. **B and C**: histograms depict ATF4 and ATF3 protein expression, respectively, corrected to β-actin in whole lung extracts. Bars represent means (n = 6–7) and error bars indicate SE. Time significantly decreased ATF4 protein expression (P < 0.05), whereas hyperoxia significantly increased ATF3 protein expression at 72 h (P < 0.001).
work in rat tissue precluded a closer examination of a role for these kinases in intact lung tissue.

Hyperoxia induces the expression of the ISR downstream target GCLC. ATF4 is known to induce the transcription of at least two genes involved redox protection, HO-1 and GCLC (27, 28). In newborn rat lungs, Ox increased GCLC mRNA expression twofold at 24 h, but not at 72 h, a pattern that mirrors the changes in nuclear ATF4 protein abundance (Fig. 8B). In contrast, we did not observe changes in HO-1 mRNA expression at either time point, in agreement with previous studies (7). Although not proof of principle, these findings are consistent with ISR-mediated induction of cytoprotective mechanisms promoting redox homeostasis.

DISCUSSION

Oxidative stress is a well-recognized activator of the UPR that has been described in human lung diseases and in animal models of human disease. Inhalation exposure of fine particulate matter induces ER stress and activates PERK and XBP1 splicing in the mouse lung as well as in the macrophage cell line RAW264.7 (24). Lungs and type II cells derived from patients with idiopathic pulmonary fibrosis also show increased ATF6 and ATF4 protein levels and significantly enhanced XBP1s mRNA expression compared with donor lungs (23). Recent studies have demonstrated that BiP and PDI colocalize

Fig. 5. Hyperoxia increases ATF3 mRNA expression. Total RNA was isolated from individual right upper lobes of RA- and Ox-exposed pups, and quantities of ATF4 (A) and ATF3 (B) relative to β-actin mRNA were determined using quantitative RT-PCR as described in METHODS. Boxes within histograms represent 25th and 75th percentiles, dashed lines indicate the median, and solid lines indicate the mean of each group. * P < 0.01, significant difference in ATF3 expression at 72 h (RA vs. Ox).

Fig. 6. Hyperoxia induces nuclear translocation and cell-specific ATF4/3 expression. A: representative immunoblots of nuclear protein content of ATF4 and ATF3. Expression of c-Jun and ERK1/2 was used to denote the purity of nuclear (N) and cytoplasmic (C) pools, respectively. Black lines within the immunoblots represent noncontiguous portions of the same gel or a different gel using identical loading and samples. B: representative images (×400) of lung sections stained for ATF4 and ATF3 (arrows indicate 3,3′-diaminobenzidine-positive cells) following exposure to 24 and 72 h of RA or Ox. Positive cells were common within the alveolar wall and were more prominent at 24 h for ATF4 and at 72 h for ATF3. C: photomicrographs (taken at ×400 and then identically cropped) depicting dual labeling of lung sections with eIF2α (Ser51) (orange) and vimentin (green) from rat pups exposed to 72 h of RA or Ox. Cells staining positive for phosphorylated eIF2α did not label with vimentin. Staining for phosphorylated eIF2α was more intense in sections from pups exposed to 95% O₂.
to lamellar bodies in rat type II cells and that mice containing a nonfunctional ER localization sequence within BiP die shortly after birth secondary to respiratory distress from impaired surfactant secretion and reduced surfactant protein C (SP-C) expression (8, 32, 33). The highly secretory nature of alveolar type II cells may render these cells particularly vulnerable to ER stress. Activation of the UPR would seem logical in response to hyperoxia, because ER stress-induced PERK activation of the UPR phosphorylates NF-E2-related factor 2 (Nrf2) in addition to eIF2\(\alpha\), promoting Nrf2 nuclear entry and the transcription of genes that promote redox homeostasis, including HO-1, glutathione S-transferase, and \(\gamma\)-glutamylcysteine synthetase (5, 34, 35).

Although hyperoxia does not appear to activate the UPR, the ability of hyperoxia to coordinately increase eIF2\(\alpha\) phosphorylation and inhibit the initiation of mRNA translation in the newborn lung raises speculation that hyperoxia may activate the ISR (22). Suppression of global protein synthesis conserves cellular energy and diverts the amino acids cysteine, glycine, and glutamate toward glutathione biosynthesis (17). In turn, the reduction in cap-dependent mRNA translation promotes the synthesis of ATF4 to induce the expression of cysteine and glycine transporters and enzymes involved in the synthesis of glutathione (17). We found that hyperoxia-induced eIF2\(\alpha\) phosphorylation is temporally related to the nuclear accumulation of ATF4 but not to alterations in ATF4 mRNA expression, findings consistent with eIF2\(\alpha\)-mediated enhancement of the translational efficiency of ATF4 mRNA. The induction of ATF4 produces multiple adaptive downstream effects. In PC12 cells, the vitamin E analog \(\alpha\)-tocopheryl quinone imparts oxidant resistance by enhancing ATF4-dependent glutathione biosynthesis (37). The ability of hyperoxia to upregulate glutathione peroxidase in cultured alveolar type II cells further suggests that ISR activation may be integral to the antioxidant response (4). In the intact newborn rat lung, ATF4 protein expression parallels the induction the ISR-response gene GCLC, a rate-limiting enzyme in glutathione biosynthesis, thereby supporting the notion that the ISR plays a protective role.

Fig. 7. Hyperoxia activates the ISR in select primary human lung cells. Representative immunoblots are from primary human lung cells exposed to 6–24 h of RA or Ox. Cells were also treated for 6 h with 4 mM thapsigargin. Thap, thapsigargin.

Fig. 8. Hyperoxia does not alter PKR phosphorylation but increases downstream ISR target gene expression. A: representative immunoblots of whole lung lysates from rat pups exposed to 24 and 72 h of RA and Ox. Arrow indicates the appropriate molecular weight of PKR (with nonspecific band above). Black lines within the immunoblots represent noncontiguous portions of the same gel. B: total RNA was isolated from individual right upper lobes, and the quantity of glutamate-cysteine ligase catalytic subunit (GCLC) relative to 36B4-actin mRNA was determined using quantitative RT-PCR. Bars represent means (\(n = 4\)) and error bars indicate SE.
Induction of ATF4 also augments the expression of the proapoptotic translation factor C/EBP homology protein (CHOP), which downregulates Bcl-2 (31). Exposure to 95–100% O2 induces CHOP mRNA expression in the bronchiolar epithelium of adult mice and in isolated type II cells in culture (36). Although we were unable to identify alterations in CHOP protein expression in O2-exposed whole lung lysates, the duration of exposure may have been insufficient to induce cell death. This is particularly important given that hyperoxia was recently shown to enhance CHOP expression after 72 h in conjunction with increased ATF4 mRNA expression (25). Given that ATF3 also has been shown to regulate CHOP expression, determining whether the alterations in pulmonary architecture in infants with BPD represents a failure of the ISR-triggered antioxidant responses or a beneficial effect of ISR-mediated CHOP induction warrants further exploration (18, 20).

Aside from CHOP, another downstream transcriptional effect of ATF4 is the CREB subfamily member ATF3. In the context of the ISR, induction of ATF3 transcription is ATF4 dependent. Loss of ATF4 prevents thapsigargin- and amino acid deprivation-stimulated ISR activation (20). In the hyperoxia-exposed newborn rat lung, the transient nuclear accumulation of ATF4 and enhanced ATF4 immunoreactivity are followed by increased ATF3 mRNA and protein expression. Expression of ATF3 is also responsive to oxidant stress signals transduced by JNK, p38 MAPK, and ERK1/2 (13). The inability to document hyperoxia-mediated phosphorylation of any of these MAPKs in the newborn lung further supports the suggestion that eIF2α-mediated events mediate the induction of ATF3 within the hyperoxic lung.

The inability of hyperoxia to activate the UPR excludes PERK as the responsible eIF2α kinase. Isolation of the hyperoxia-activated eIF2α kinase is challenging because there are few reliable markers for HRI, GCN2, or PKR activation in intact tissue. Oxidative stress has been shown to activate HRI, GCN2, and PKR under specific conditions, raising the possibility that multiple kinases may be activated depending on the duration of exposure and the reactive species generated (38, 39). A recent publication implicates PKR as the kinase responsible for hyperoxia-mediated ATF4 and CHOP induction in MLE-12 cells (25). We were unable to detect changes in the phosphorylation of PKR on Thr446 in hyperoxia-treated rat pups or CHOP expression despite detecting increased expression of the latter in tunicamycin-treated rat fibroblasts and epithelial cells in culture (not shown). Likewise, we could not detect O2-induced alterations in the phosphorylation of PKR or GCN2 in HSAEC or PIMEC despite clear evidence of ISR activation. These findings suggest that the hyperoxic response in newborns may be unique or that a kinase-specific phoshathase subunit, such as GADD34 (growth arrest and DNA damage-inducible protein), or a kinase-specific inhibitor, such as MetAP2/p67 (methionine aminopeptidase 2), functions to enhance eIF2α phosphorylation (6).

One proposal defines ATF3 as a “hub of the cellular adaptive-response network” modulating the inflammatory response (13). Induction of ATF3 occurs in response to a wide range of Toll-like receptors, which function as negative regulators of TLR-4-induced inflammation (12, 43). During ovalbumin challenge in mice, ATF3 deficiency significantly increases airway hyperresponsiveness, pulmonary eosinophilia, and expression of the cytokines IL-4, IL-5, and IL-13 and the chemokines CCL2, CCL7, CXCL1, and CCL2 (11). Cyclic stretch also induces ATF3 expression in BEAS-2B lung epithelial cells and parenchymal lung cells of rats ventilated with high tidal volumes (1). Increased pulmonary ATF3 expression is found in the midgestational baboon model of BPD (21). In pancreatic islets, however, ATF3 serves a proapoptotic and proinflammatory role by upregulating the expression of IL-1β, IL-6, TNF-α, and CCL-2 (45). Determining whether hyperoxia-induced ATF3 expression in the lung is ultimately protective or deleterious awaits further study.

Placed within the context of our previous work, the observations of the current study strongly suggest that hyperoxia-induced reductions in global pulmonary protein synthesis and increased eIF2α phosphorylation are markers of ISR activation (22). It is tempting to speculate that ISR-mediated events are integral to the adaptation to hyperoxia as implied by the enhanced GCLC expression. Further exploration of the precise roles of ATF3 and ATF4 in the regulation of redox balance, immune function, and cell survival are necessary to confirm this theory and to delineate how ISR signaling might be manipulated to minimize O2-induced lung injury in humans.

DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: W.M.K. and J.S.S. conception and design of research; W.M.K., L.Z., Y.W., and J.S.S. performed experiments; W.M.K., L.Z., Y.W., and J.S.S. analyzed data; W.M.K. and J.S.S. interpreted results of experiments; W.M.K. and J.S.S. drafted manuscript; W.M.K., L.Z., Y.W., and J.S.S. approved final version of manuscript; J.S.S. prepared figures; J.S.S. edited and revised manuscript.

REFERENCES

HYPEROXIA ACTIVATES ISR


