Published in final edited form as:

Exp Lung Res. 2016 April ; 42(3): 121–130. doi:10.3109/01902148.2016.1157712.

PRIOR HYPOXIA PREVENTS DOWNREGULATION OF ACE-2 BY HYPEROXIA IN FETAL HUMAN LUNG FIBROBLASTS

Tarek L. Mohamed¹, Hang T. Nguyen², Amal Abdul-Hafez¹, Vinh X. Dang³, MyTrang T. Dang⁴, Ira H. Gewolb¹, and Bruce D. Uhal³

¹Department of Pediatrics/Neonatology, Michigan State University, East Lansing, Michigan 48824

²Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan 48824

³Department of Physiology, Michigan State University, East Lansing, Michigan 48824

⁴Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, Michigan 48824

Abstract

Purpose/Aim of Study—The renin angiotensin system is involved in experimentally induced lung fibrosis. Angiotensin (ANG)-II is profibrotic. Angiotensin converting enzyme-2 (ACE-2) cleaves ANG-II and is thus protective. ACE-2 has recently been reported to be significantly decreased under hyperoxic conditions. Hyperoxia is linked to Bronchopulmonary Dysplasia and lung fibrosis. Fetal lung cells normally do not undergo fibrotic changes with physiologic hypoxemia. We hypothesized that hypoxia prior to hyperoxic exposure in fetal lung fibroblasts (IMR-90 cell line) might be protective by preventing ACE-2 down-regulation.

Materials and Methods—IMR-90 cells were exposed to hypoxia (1% O₂/99% N₂) followed by hyperoxia (95% O₂/5% CO₂) or normoxia (21% O₂) in vitro. Cells and culture media were recovered separately for assays of ACE-2, TNF- α Converting Enzyme (TACE), α Smooth muscle actin “ α SMA” -myofibroblast marker-, N-Cadherin and β -catenin immunoreactive protein.

Results—ACE-2 significantly increased when IMR-90 were hypoxic prior to hyperoxic exposure with no recovery. In contrast to hyperoxia alone, ACE-2 did not decrease when IMR-90 were hypoxic prior to hyperoxic exposure with recovery. TACE/ADAM17 protein and mRNA were significantly increased in cells allowed to recover in 21% O₂. α SMA N-Cadherin and β -Catenin proteins were significantly decreased in both groups.

Conclusions—Hypoxia prior to hyperoxic exposure of fetal lung fibroblasts prevented ACE-2 downregulation and decreased ADAM17/TACE protein and mRNA. α SMA, N-Cadherin and β -catenin were also significantly decreased under these conditions.

Address all correspondence and reprint requests to: Bruce D. Uhal, Ph.D., Department of Physiology, Michigan State University, 3197 Biomedical and Physical Sciences Building, East Lansing, MI 48824, Phone: (517) 884-5131, Fax: (517) 355-5125, uhal@msu.edu.

Authors have nothing to disclose.

This manuscript has not been published elsewhere and it has not been submitted simultaneously for publication elsewhere.

DECLARATION OF INTERESTS: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Keywords

Chronic Lung Disease; Bronchopulmonary Dysplasia; hypoxia; hyperoxia; fetal lung fibroblasts

INTRODUCTION

The human fetus depends upon the placenta for gas and nutrient exchange with the maternal circulation. The intrauterine oxygen tension is low compared to that seen in extrauterine life. The highest oxygenated fetal blood is found in the umbilical vein, with PaO_2 of 55 ± 7 mmHg (1). Oxygen saturation decreases when mixed with venous return, so that blood returning to the placenta will have a P_aO_2 of 15-25 mmHg. Despite low oxygen tension, there is adequate tissue oxygenation (2) and under normal conditions, in spite of the physiologic hypoxemia, lung cells do not evince fibrotic changes at birth or progress to chronic lung disease. This is surprising in light of our earlier work, which showed that Hypoxia Inducible Factor-1 α (HIF-1 α) is required in the signaling by which TGF- β 1 activates the profibrotic gene angiotensinogen (AGT) in fetal human lung fibroblasts (3). During hypoxia, HIF-1 α is stabilized and regulates various genes, such as those involved in angiogenesis or oxygen transport (4).

Related work from our laboratory has shown that Angiotensin Converting Enzyme (ACE)-2, which degrades Angiotensin (ANG) II and is thereby protective against lung fibrosis, is down-regulated in human interstitial pulmonary fibrosis (IPF) and in experimentally-induced lung fibrosis (5). The main function of ACE-2 is to convert the octapeptide Angiotensin II to the heptapeptide angiotensin 1-7 (ANG 1-7). This limits the accumulation of ANG II and protects against experimental lung fibrosis. A comprehensive review of the literature reveals a strong association between exposure to hypoxia and lung fibrosis. Previous work from this laboratory has shown that in tissue samples from adult lung IPF, the induction of fibrosis in lung injury is associated with the angiotensin system (5-6). In animal models, activation of the angiotensin system is both necessary and sufficient to induce epithelial cell fibrosis. This activation involves down-regulation of ACE-2 (which degrades ANG II), with subsequent accumulation of the profibrotic ANG II. We have also demonstrated that ACE-2 inhibitors such as DX 600 will also lead to fibrosis (5).

Although the terms hypoxia and hypoxemia are often used interchangeably, they are not synonymous. Hypoxemia is defined as a condition where arterial oxygen tension (PaO_2) is below normal (normal PaO_2 in neonates = 50–70mmHg). Hypoxia is defined as the failure of oxygenation at the tissue level. Hypoxia and hypoxemia may or may not occur together. Generally, the presence of hypoxemia suggests hypoxia. However, hypoxia may not be present in patients with hypoxemia if the patient compensates for a low PaO_2 by increasing oxygen delivery. This is typically achieved by increasing cardiac output or decreasing tissue oxygen consumption. Conversely, patients who are not hypoxemic may be hypoxic if oxygen delivery to tissues is impaired or if tissues are unable to use oxygen effectively. Nevertheless, hypoxemia is by far the most common cause of tissue hypoxia (7).

Bronchopulmonary Dysplasia (BPD) is a major cause of morbidity and mortality during the first year of life, and can lead to significantly increased airway reactivity and obstructive

airway disease (8). Nowadays, the “new BPD” is characterized by simplification and arrest of development of alveoli and less overt fibrosis, as compared to the “old BPD” which is characterized by more overt fibrosis (9-10)

Hypoxia induces lung injury and may contribute to BPD. A review of the literature reveals a strong association between exposure to hypoxia and subsequent chronic lung disease. Rassler and co-workers (11) have investigated the effects of normobaric hypoxia on rat lungs and hypothesized that the hypoxic exposure would induce lung injury by development of fibrosis. Rats were exposed to 10% O₂ over 6-168 h. They analyzed cardiovascular function and pulmonary changes, lung histology and mRNA expression of extracellular matrix (ECM) molecules in the lung. Significant hemodynamic changes occurred after 168 h of hypoxic exposure. Moderate pulmonary edema appeared after 8 h and peaked after 16 h of hypoxia, accompanied by fibrosis and vascular hypertrophy. mRNA expression of transforming growth factor- β -2 and - β -3 was up-regulated in lung tissue after 8 h of hypoxia. After 8-16 h, mRNA expression of collagen types I and III and of other ECM molecules was significantly elevated and increased further with longer exposure to hypoxia. The time course of hypoxia-induced pulmonary injury resembled that previously observed after continuous norepinephrine infusion in rats (11).

Tzouveleakis and co-workers (12) performed expression profiling of disease progression in a well-characterized animal model of BPD. Differentially expressed genes that were identified were compared with all publicly available expression profiles both from human patients and animal models. The role of HIF-1 α in disease pathogenesis was examined with a series of immunostainings, both in the animal model as well as in tissue microarrays containing tissue samples of human patients, followed by computerized image analysis. The role of HIF-1 α signaling was further explored and revealed HIF-1 α overexpression in the hyperplastic epithelium of fibrotic lungs, co-localized with its target genes p53 and VEGF.

Among the criteria used to assess epithelial injury, which include accumulation of products of lipid peroxidation, enhancement of pulmonary microvascular permeability and morphological changes, monitoring of ACE-2 is of great importance because ACE-2 is associated with the epithelial surface and thus reflects epithelial status. It has been demonstrated that lung epithelial injury involves the angiotensin system (6, 13-15).

In previous work from this laboratory, Oarhe et al. (16) recently showed that ACE-2 is expressed in fetal human lung fibroblasts and is significantly down-regulated by exposure to hyperoxic gas followed by normoxic recovery through a shedding mechanism mediated by Tumor Necrosis Factor-Alpha Converting Enzyme (ADAM17/TACE). We therefore hypothesized that hypoxia prior to hyperoxia in fetal lung fibroblasts may be protective (prevent ACE-2 from decreasing), simulating the physiologic fetal hypoxemia prior to be exposed to the normoxia/relative hyperoxia after birth.

MATERIALS AND METHODS

The fetal lung fibroblast IMR 90 cell line was purchased from ATCC (Manassas, VA). 6-well collagen I coated plates were obtained from BD Biosciences (Bedford, MA). ACE-2

antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). α SMA, NCadherin, β -catenin and β -actin antibodies were purchased from Cell Signaling Technology (Danvers, MA). Oxygen delivery was confirmed in the hypoxia/hyperoxia chamber outlet using a Maxtec MaxO2+A Oxygen Analyzer - R217P62 (Salt Lake City, Utah). Protease Inhibitor Cocktail P840 was purchased from Sigma (Saint Louis, MO) and the Pierce BCA Protein Assay Kit from Thermo Fisher Scientific Pierce Biotechnology (Rockford, IL), 10X Tris/glycine/SDS buffer was obtained from BioRad (Berkeley, CA), and HRP-conjugated goat anti-rabbit secondary antibody from Santa Cruz Biotechnology (Santa Cruz, CA). All other materials were of reagent grade.

Cell Culture

This study was approved by the Institutional Review Board of Michigan State University, East Lansing, MI. Early passages (15 or less) of the fetal lung fibroblast IMR 90 cell line were sub-cultured in complete media with 10% Fetal Bovine Serum (FBS). Cells were harvested at confluence and assayed for ACE-2, TACE, α SMA, N Cadherin, β catenin and β -actin using SDS-PAGE and immunoblotting (Western Blotting). IMR-90 cells were allowed to grow to confluence in 6-well collagen I coated plates and then exposed to hypoxia (1% O₂ + 99% N₂) for 24 hours in 5% FBS, Bicarbonate-free Minimum Essential Medium (MEM) with HEPES (4-2-hydroxyethyl-1-piperazineethanesulfonic acid) buffer followed by hyperoxia (95% O₂ + 5% CO₂) for 72 hours in 5% FBS- complete MEM, then either harvested immediately or allowed to “recover” in 21% O₂ + 5% CO₂ in serum free MEM. Control cells were exposed to 21% O₂ in media corresponding to the treated group (i.e. during first 24 hours controls were in 5% FBS Bicarbonate free MEM with HEPES buffer followed by 5% FBS MEM for 72 hours then either harvested immediately, or the media was aspirated and cells were rinsed once with serum free medium and then cultured in serum free MEM for 24 hours. All cells were harvested, on ice, with protease inhibitor cocktail.

The hypoxia/hyperoxia chamber was flushed with the appropriate treatment gas, at a flow of 3 LPM for 45-60 min, after which the flow was decreased to 0.5 LPM until the oxygen analyzer indicated that the desired oxygen level had been reached. Each well was filled with 10ml of medium to ensure no changes in solute concentration or pH after the treatment period.

Cell Viability

Viability was assessed using Trypan Blue. A minimum of 1000 cells per well were counted in at least three separate microscopic fields. These counts were conducted in at least three culture wells per treatment group; results were compiled and analyzed using InStat statistical software (GraphPad Software, Inc., San Diego, California).

Western blotting

After performing a protein assay using the BCA method (Pierce, Grand Island, NY), 45 μ g of protein lysate and protease inhibitor cocktail were loaded in each well of 10% Tris HCL polyacrylamide gels, and separated by SDS-PAGE, in 10X Tris/glycine/SDS buffer. Then proteins are transferred to Polyvinylidene Difluoride “PVDF” blotting membrane “solid

phase support,” then blocked by 5% nonfat dry milk in 0.1% tween 20 in Tris-buffered saline.

Western blot analysis was performed using polyclonal antibody against ACE-2, TACE, α SMA, N-Cadherin and β -catenin. β -actin was used to normalize the assays. Bands were visualized by HRP-conjugated goat anti-rabbit secondary antibody using enhanced chemiluminescence detection by standard film techniques

RNA isolation and Real-time PCR for Tumor Necrosis Factor-Alpha Converting Enzyme (TACE) and A-Disintegrin and Metalloproteinase domain-containing protein-17 (ADAM-17)

After treatment, IMR 90 cells were extracted for total RNA using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. First-strand cDNA was synthesized from total RNA using Superscript II reverse transcriptase and oligo (dT)₁₂₋₁₈ (Invitrogen). Real-time PCR was carried out with cDNA synthesized from 50 ng of total RNA, (SYBR Green PCR core reagents (Applied Biosystems, Foster City, CA)) according to the manufacturer's instructions, and 0.2 μ M specific primers for human ADAM17/TACE (sense 5'-TTG GTG GTA GCA GAT CAT CG-3' and antisense 5'-CTG GGA GAG CCA ACA TAA GC-3') and GAPDH (Sense 5'-CCC CTT CAT ACC CTC ACG TA-3' and Antisense 5'-ACA AGC TTC CCG TTC TCA G-3'). The thermal profile started with 10 min activation at 95°C followed by 40 cycles of denaturation at 95°C for 60 s, annealing at 55°C for 60 s, extension at 72°C for 60 s, and ending with dissociation curve analysis of the PCR products. Reactions were performed in a StepOnePlus Real-time PCR system instrument (Applied Biosystems). Threshold cycle (CT) data were collected using StepOne software version 2.1 (Applied Biosystems). The relative TACE/ADAM-17 expression was normalized to GAPDH and calculated with the comparative CT method of 2^{-CT} , where $CT = CT_{ACE-2} - CT_{GAPDH}$ and $CT = CT_{treatment} - CT_{control}$.

Results

Effect of hypoxia prior to hyperoxia on plasma membrane integrity in IMR-90 cells

Figure 1 depicts the loss of plasma membrane integrity with hypoxia prior to hyperoxic exposure of the IMR90 cell line. Without normoxic recovery, exposure to hypoxic or hyperoxic gases had no significant effect on cell viability. With normoxic recovery however, the prior exposure to hypoxia significantly decreased the number of trypan blue-positive cells induced by hyperoxic gas (right two bars).

Effect of hypoxia prior to hyperoxia on ACE-2 in IMR-90 cells

Figure 2 shows that treatment of fetal lung fibroblast IMR-90 cells with hypoxia prior to hyperoxic exposure significantly up-regulates ACE-2, if cells are analyzed at the end of the hyperoxic exposure. If a 24h period of normoxic recovery follows the hyperoxic exposure, ACE-2 immunoreactive protein is unchanged relative to unexposed cells; this further clarifies the work of Oarhe et al. (16) which showed a reduction of ACE-2 following hyperoxic exposure alone.

Effect of hypoxia prior to hyperoxia on TACE/ADAM17 in IMR-90 cells

In Figure 3, treatment of fetal lung fibroblast IMR-90 cells with hypoxia prior to hyperoxic exposure (followed by normoxic recovery) down-regulated TACE/ADAM17 as assessed using Western blotting (Panel A). By densitometry (Panel B), the decrease was statistically significant ($p<0.05$). In Panel C, TACE/ADAM17 mRNA was also reduced by when exposure to hypoxia preceded exposure to hyperoxia, in contrast to the increase in TACE mRNA caused by hyperoxic gas alone (16).

Effect of hypoxia prior to hyperoxia on cell differentiation marker expression by IMR-90 cells

Figure 4 shows that alpha-Smooth Muscle Actin (α SMA), the standard marker for myofibroblast transition, is significantly down-regulated in fetal lung fibroblasts by hypoxia prior to hyperoxic exposure.

Figures 5 and 6 show that both N-Cadherin and β -Catenin, respectively, are also significantly downregulated. The downregulation was observed both with and without normoxic recovery.

Schematic diagram showing effect of hyperoxia alone versus hypoxia preceding hyperoxia on TACE/ ADAM17 and ACE-2

Figure 7 shows the effect of hyperoxia in down-regulating TACE/ADAM17, thus down-regulating ACE-2 through shedding of its ectodomain, and leading to an accumulation of the profibrotic ANG II, in turn decreasing the antifibrotic ANG 1-7, finally leading to fibrosis. Hypoxia preceding hyperoxia, in contrast, up-regulates TACE/ADAM17, thus preventing ACE-2 down-regulation, allowing clearance of the profibrotic ANG II, increasing the antifibrotic ANG 1-7, and thus theoretically protecting from fibrosis (panel A). Hyperoxia followed by normoxic recovery of IMR-90 cells, results in an increase in TACE and a decrease in protective ACE-2, leading to fibrosis (panel B). Hypoxia prior to hyperoxic exposure followed by normoxic recovery leads to a decrease in TACE, in turn preventing ACE-2 from decreasing, which may protect against fibrosis (Panel C).

Discussion

Barotrauma via mechanical ventilation and hyperoxia are well studied pathogenetic factors in BPD. The renin-angiotensin system's role in lung injury has been well studied (17-18). It has been demonstrated that ANG II is a potential pulmonary profibrotic mediator that stimulates collagen synthesis through ANG II type I receptor activation. The ANG II receptor blocker losartan attenuates experimental-induced pulmonary fibrosis (5). There are also counter regulatory mediators-- ACE-2 and ANG 1-7, as well as its receptor Mas (19). The "protective" ACE-2 is noted to be down-regulated in patients with IPF as well as in experimentally-induced lung fibrosis (5). The effect of the renin-angiotensin system in initiating and causing fibrosis has been demonstrated not only in lung tissue, but also in retina, pancreas, heart, kidney and liver (15-20-23).

It has been reported that the ACE-2 knock out mouse experimental lung injury model has more severe lung disease (23), and that treating wild as well as ACE-2 knock out mouse

models of lung injury with recombinant ACE 2 protein improves lung injury symptoms (24). Recombinant ACE-2 was demonstrated to improve pulmonary blood flow and oxygenation in lipopolysaccharide-induced lung injury in piglets (18). Recombinant human ACE-2 has been tested in healthy individuals in clinical trials to determine medication pharmacokinetics and pharmacodynamics (25), and is being investigated by GlaxoSmithKline (“pipeline drug GSK2586881”) in a clinical trial to treat adult acute lung injury.

The potential toxicity of hyperoxia has been well-studied. In 1968, Northway and Rosan (26) demonstrated that supplemental oxygen can cause Bronchopulmonary Dysplasia (BPD) which in turn can lead to Chronic Lung Disease (CLD). Oxygen is frequently used in NICUs for pulmonary insufficiency in preterm babies. The new BPD, characterized by alveolar simplification and arrest of development, was demonstrated after prolonged exposure to hyperoxia in a neonatal mouse model (27).

Ratner et al. (27) demonstrated that; cycling hypoxia with hyperoxia will exacerbate lung injury. However, the intermittent hypoxia group of mice (no hyperoxia) did not show any change in protein carbonyl or radial alveolar count compared to normoxic controls (markers of oxidative stress and lung injury). Interestingly, total/oxidized glutathione ratio in the hypoxic group also did not show any change compared to controls, suggesting that hypoxic mice did not have any BPD-like changes, and that hypoxia alone did not affect the antioxidant capacity.

Recent data from our lab (16) showed that ACE-2 is expressed but down-regulated in IMR-90 cells exposed to hyperoxia followed by normoxic recovery. Normally, in spite of hypoxemia in-utero, fetal lung cells do not undergo fibrotic changes and/or develop changes of chronic lung disease. On this basis, it was hypothesized that hypoxia prior to hyperoxia in fetal lung fibroblasts may be protective (prevent ACE-2 from decreasing). This finding suggests that ACE-2 might be potential future therapy for babies at risk for developing BPD.

Oarhe et al. (16) recently reported that hyperoxia followed by normoxic recovery worsens plasma membrane integrity compared to controls. With hypoxia preceding hyperoxic exposure, we noted a significant decrease in mean number of dead cells in the treated group followed by normoxic recovery, when compared to corresponding controls (in 21% the whole time). There was no change between treated and corresponding control in the non-recovery group (Figure 1). Investigating potential Redox poise/glutathione antioxidant may be germane.

The antifibrotic, protective enzyme ACE-2 significantly increased when fetal lung fibroblasts were hypoxic prior to hyperoxic exposure with no normoxic recovery. ACE-2 did not decrease when fetal lung fibroblasts were hypoxic prior to hyperoxic exposure with normoxic recovery group (Figure 2). In Figure 3, TACE/ADAM17 immunoreactive protein and mRNA were significantly decreased when fetal lung fibroblasts were hypoxic prior to hyperoxic exposure (with normoxic recovery), or did not change (without recovery, data not shown). Given that TACE/ADAM17 *increased* in our previous study (16) in response to hyperoxia alone, this might explain why ACE-2 did not decrease when hypoxia preceded the

hyperoxic exposure, although we were not able to show such a causation experimentally here.

Oarhe et al. (16) showed that ACE-2 is expressed but down-regulated in IMR-90 cells exposed to hyperoxia followed by normoxic recovery. This ACE-2 down-regulation was attributed to shedding by the up-regulated TACE/ADAM17 both at protein and mRNA levels, suggesting that TACE regulation might occur at the transcriptional level for both hypoxia and hyperoxia exposures. By adding 24 hours of hypoxia prior to the hyperoxia, there was a reversal of the pro-fibrotic condition (decreasing ACE-2 and increasing TACE/ADAM17), to a more protective condition. TACE increase will lead to an ACE-2 decrease by increasing shedding; however a decrease in TACE might not necessarily lead to an increase in ACE-2 which may be related to other factors.

Developing lungs normally have myofibroblasts along terminal airways. With lung injury, myofibroblasts increase in number and terminal air spaces increase; this is mediated by TGF- β . Toti et al. (28) has shown that autopsies from premature babies with RDS showed proliferation and migration of myofibroblasts at sites of lung injury during the process of lung repair, which suggests that myofibroblasts play a role in premature lung repair. Lungs of premature babies with BPD and CLD have been shown to have increased myofibroblasts (29).

We noted that α -SMA immunoreactive protein, a marker of the transition of fibroblast to myofibroblast, was significantly decreased when fetal lung fibroblasts were hypoxic prior to hyperoxic exposure in both recovery and non-recovery groups.

Earlier works suggested that epithelial-to-mesenchymal transition (EMT) occurs during fibrogenesis in a number of organs, including the lungs (30-31). Our study demonstrated a significant decrease in the mesenchymal markers (α -SMA, N-Cadherin and β -catenin) in response to hypoxia preceding hyperoxia, suggesting that hypoxia prior to hyperoxia might be a protective mechanism. Whether similar EMT responses occur in response to challenging of the alveolar epithelial cells to hypoxia preceding hyperoxia remains to be determined.

We speculate that adult epithelial lung cells will not have the same protective mechanisms (including a reduction in ACE-2) that the fetal lung fibroblast has, possibly explaining why the elderly are more at risk for lung fibrosis.

In summary, this study showed that ACE-2 is expressed by fetal lung fibroblasts. With hypoxia preceding hyperoxic exposure of cultured fetal lung fibroblasts, we noted an increase in ACE-2 protein, a decrease in TACE/ADAM17 protein and mRNA, and in α SMA, N-Cadherin and β -catenin. The recently shown effect of hyperoxia on ACE-2 and TACE (16) has been reversed by hypoxia prior to hyperoxic exposure. The mechanism whereby this protection occurs is still unknown; however redox poise may play a part. We are not advocating exposure of newborns to hypoxia; however, this study sheds some light on understanding the transition from the hypoxemic environment in-utero to the relative hyperoxic environment in postnatal life. The role of ACE-2 in neonatal lung injury, may be

germane to adult lung disease as well, since “protective” ACE-2 levels may decline with age, thus explaining why the elderly are more at risk for lung fibrosis.

ACKNOWLEDGMENTS

Portions of the work conducted by H.N was conducted in partial fulfillment of the requirements for the degree Doctor of Philosophy from Michigan State University.

FUNDING

This work was supported by American Academy of Pediatrics Marshal Klaus award to T.M., HL-45136 to B.D.U. and by a grant from the Sparrow Hospital Fellowship Research Fund (to T.M.).

REFERENCES

1. Soothill PW, Nicolaides KH, Rodeck CH, Gamsu H. Blood gases and acid-base status of the human second-trimester fetus. *Obstet Gynecol.* 1986; 68:173–6. [PubMed: 3090491]
2. Goldsmith, JP. Delivery room resuscitation of the newborn.. In: Martin, RJ.; Fanaroff, AA.; Walsh, MC., editors. *Neonatal-Perinatal Medicine: Diseases of the Fetus and Infant.* Elsevier Mosby; St. Louis, USA: 2011. p. 449-58.
3. Abdul-Hafez A, Shu R, Uhal B. JunD and HIF-1 α mediate transcriptional activation of angiotensinogen by TGF- β 1 in human lung fibroblasts. *FASEB J.* June. 2009; 23(6):1655–62. [PubMed: 19211927]
4. Semenza GL. HIF-1 and human disease: one highly involved factor. *Genes Dev.* 2000; 14:1983–91. [PubMed: 10950862]
5. Li X, Molina-Molina M, Abdul-Hafez A, Uhal V, Xaubet A, Uhal B. Angiotensin converting enzyme-2 is protective but downregulated in human and experimental lung fibrosis. *Am J Physiol Lung Cell Mol Physiol.* Jul. 2008; 295:L178–85. [PubMed: 18441099]
6. Li X, Zhang H, Soledad-Conrad V, Zhuang J, Uhal BD. Bleomycin induced apoptosis of alveolar epithelial cell requires angiotensin synthesis de novo. *Am J Physiol lung Cell Mol Physiol.* 2003; 284:L501–7. [PubMed: 12573988]
7. Jacob, S.; Cory, F. *Common Surgical Diseases.* Springer; New York: 2008. Hypoxemia and hypoxia.; p. 391-94.
8. Lemons JA, Bauer CR, Oh W, Korones SB, Papile LA, Stoll BJ, et al. Very low birth weight outcomes of the National Institute of Child health and human development neonatal research network, January 1995 through December 1996. *NICHD Neonatal Research Network. Pediatrics.* 2001; 107:E1. [PubMed: 11134465]
9. Mosca F, Colnaghi M, Fumagalli M. BPD: old and new problems. *J Matern Fetal Neonatal Med.* 2011; 1:k80–2.
10. Vento G, Capoluongo E, Matassa PG, et al. Serum levels of seven cytokines in premature ventilated newborns: correlations with old and new forms of bronchopulmonary dysplasia. *Intensive Care Med.* 2006; 32:723–30. [PubMed: 16550369]
11. Rassler B, Marx G, Reissig C, Rohling MA, Tannapfel A, Wenger RH, et al. Time course of hypoxia-induced lung injury in rats. *Respir Physiol Neurobiol.* 2007; 15:45–54. [PubMed: 17597012]
12. Tzouveleakis A, Harokopos V, Paparountas T, Oikonomou N, Chatziioannou A, Vilaras G, et al. Comparative expression profiling in pulmonary fibrosis suggests a role of hypoxia-inducible factor-1 α in disease pathogenesis. *Am J Respir Crit Care Med.* 2007; 11:1108–19. [PubMed: 17761615]
13. Wang R, Zagariya A, Ang E, Ibarra-Sunga O, Uhal BD. Fas-induced apoptosis of alveolar epithelial cells requires angiotensin II generation and receptor interaction. *Am J Physiol lung Cell Mol Physiol.* 1999; 277:L1245–50.
14. Klahr S, Morrissey JJ. Comparative study of angiotensin converting enzyme inhibitors and angiotensin II receptor antagonists in interstitial scarring. *Kidney Int. Suppl.* 1997; 63:S111–4. [PubMed: 9407436]

15. Wang R, Alam G, Zagariya A, Gidea C, Pinillos H, Lalude O, et al. Apoptosis of lung epithelial cells in response to TNF-alpha requires angiotensin II generation de novo. *J cell Physiol.* 2000; 185:253–9. [PubMed: 11025447]
16. Oarhe C, Dang V, Dang M, Nguyen H, Gopallawa I, Gewolb IH, et al. Hyperoxia downregulates angiotensin-converting enzyme-2 in human fetal lung fibroblasts. *Pediatr Res.* 2015; 77:656–62. [PubMed: 25665060]
17. Marshall RP, Gohlke P, Chambers RC, Howell DC, Bottoms SE, Unger T, et al. Angiotensin II and the fibroproliferative response to acute lung injury. *Am J Physiol.* 2004; 286:L156–64.
18. Trembl B, Neu N, Kleinsasser A, Gritsch C, Finsterwalder T, Geiger R, et al. Recombinant angiotensin-converting enzyme 2 improves pulmonary blood flow and oxygenation in lipopolysaccharide-induced lung injury in piglets. *Crit Care Med.* 2010; 38:596–601. [PubMed: 19851091]
19. Shenoy V, Ferreira AJ, Qi Y, Fraga-Silva RA, Díez-Freire C, Dooies A, et al. The angiotensin-converting enzyme 2/angiogenesis-(1-7)/Mas axis confers cardiopulmonary protection against lung fibrosis and pulmonary hypertension. *Am J Respir Crit Care Med.* 2010; 182:1065–72. [PubMed: 20581171]
20. Weber KT, Sun Y. Recrutable ACE and tissue repair in the infarcted heart. *J Renin Angiotensin Aldosterone Syst.* 2000; 1:295–303. [PubMed: 11967815]
21. Yoshiji H, Kuriyama S, Yoshii J, Ikenaka Y, Noguchi R, Nakatani T, et al. Angiotensin-II type 1 receptor interaction is a major regulator for liver fibrosis development in rats. *Hepatology.* 2001; 34:745–50. [PubMed: 11584371]
22. Wang R, Ibarra-Sunga O, Verlinski L, Pick R, Uhal BD. Abrogation of bleomycin-induced epithelial apoptosis and lung fibrosis by captopril or by a caspase inhibitor. *Am J Physiol Lung Cell Mol Physiol.* 2000; 279:L143–51. [PubMed: 10893213]
23. Imai Y, Kuba K, Rao S, Huan Y, Guo F, Guan B, et al. Angiotensin-converting enzyme 2 protects from severe acute lung failure. *Nature.* 2005; 436:112–6. [PubMed: 16001071]
24. Imai Y, Kuba K, Ohto-Nakanishi T, Penninger J. Angiotensin converting enzyme II in Disease Pathogenesis. *Nature.* 2005; 436:112–6. [PubMed: 16001071]
25. Haschke M, Schuster M, Poglitsch M, Loibner H, Salzberg M, Bruggisser M, et al. Pharmacokinetics and pharmacodynamics of recombinant human angiotensin-converting enzyme 2 in healthy human subjects. *Clin Pharmacokinet.* 2013; 9:783–92. [PubMed: 23681967]
26. Northway W, Rosan R. Radiographic Features of Pulmonary Oxygen Toxicity in the Newborn: Bronchopulmonary Dysplasia. *Radiology.* 1968; 91:49–58. [PubMed: 4871379]
27. Ratner V, Slinko S, Utkina-Sosunova I, Starkov A, Polin RA, Ten VS. Hypoxic stress exacerbates hyperoxia-induced lung injury in a neonatal mouse model of bronchopulmonary dysplasia. *Neonatology.* 2009; 95:299–305. [PubMed: 19052476]
28. Toti P, Buonocore G, Tanganelli P, Catella AM, Palmeri ML, Vatti R, et al. Bronchopulmonary dysplasia of the premature baby: an immunohistochemical study. *Pediatr Pulmonol.* 1997; 24:22–28. [PubMed: 9261849]
29. Popova AP, Bozyk PD, Bentley JK, Linn MJ, Goldsmith AM, Schumacher RE, et al. Isolation of tracheal aspirate mesenchymal stromal cells predicts bronchopulmonary dysplasia. *Pediatrics.* 2010; 5:1127–33.
30. Kim KK, Kugler MC, Wolters PJ, Robillard L, Galvez MG, Brumwell AN, et al. Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix. *Proc. Natl. Acad. Sci. U.S.A.* 2006; 103(35):13180–5. [PubMed: 16924102]
31. Tanjore H, Cheng DS, Degryse AL, Zoz DF, Abdolrasulnia R, Lawson WE, et al. Alveolar epithelial cells undergo epithelial-to-mesenchymal transition in response to endoplasmic reticulum stress. *J Biol Chem.* 2011; 286:30972–80. [PubMed: 21757695]

Figure 1. Loss of plasma membrane integrity with hypoxia prior to hyperoxic exposure of IMR90 cells

The Y-axis is the mean of three determinations of dead cell number as recognized by trypan blue dye. *= $P < 0.001$ vs. control no recovery and **= $P < 0.001$ vs. corresponding control by ANOVA and Tukey-Kramer multiple comparisons test.

Figure 2. Upregulation of Angiotensin Converting Enzyme-2 (ACE-2) in fetal lung fibroblast IMR-90 cells by hypoxia prior to hyperoxic exposure

A) ACE-2 and β -actin protein were analyzed by SDS-PAGE and immunoblotting with and without recovery and their corresponding controls in 21% oxygen. B) Densitometry of ACE-2 protein fold change, normalized to β -actin protein in non-recovery group with its corresponding control. * $P < 0.01$ vs. 21% O₂.

Figure 3. Downregulation of Tumor Necrosis Factor-Alpha Converting Enzyme/ A-Disintegrin and metalloproteinase domain-containing protein 17 (TACE/ADAM-17)

A) TACE/ADAM-17 and β -actin protein by SDS-PAGE and immunoblotting for recovery group and its corresponding control (21% oxygen). B) Densitometry of TACE/ADAM-17 protein fold change, normalized to β -actin protein in recovery group with its corresponding control. * $P < 0.01$ vs. 21% O₂. C) mRNA fold-change in TACE/ADAM-17 normalized to GAPDH mRNA in recovery group compared with its corresponding control. * $P < 0.01$ vs. 21% O₂.

Figure 4. Downregulation of α Smooth Muscle Actin (αSMA) in IMR-90 cells by hypoxia prior to hyperoxic exposure

A) αSMA and β-actin protein by SDS-PAGE and Western Blotting for non-recovery group and its corresponding control and densitometry of αSMA protein fold change, normalized to β-actin protein. * $P < 0.01$ vs. 21% O₂. B) αSMA and β-actin protein in the recovery group and its corresponding control and densitometry of αSMA protein fold change, normalized to β-actin protein. *** $P < 0.0001$ vs. 21% O₂.

Figure 5. Downregulation of N-Cadherin in Fetal lung fibroblast IMR-90 cells by hypoxia prior to hyperoxic exposure

A) N-Cadherin and β -actin protein by SDS-PAGE and Western Blotting for the non-recovery group and its corresponding control and densitometry of N-Cadherin protein fold change, normalized to β -actin protein. * $P < 0.05$ vs. 21% O₂. B) N-Cadherin and β -actin protein by Western Blotting for the recovery group and its corresponding control and densitometry of NCadherin protein fold change, normalized to β -actin protein. *** $P < 0.0001$ vs. 21% O₂.

Figure 6. Downregulation of β-Catenin in Fetal lung fibroblast IMR-90 cells by hypoxia prior to hyperoxic exposure

A) β-Catenin and β-actin protein by SDS-PAGE and Western Blotting for the non-recovery group and its corresponding control and densitometry of β-Catenin protein fold change, normalized to β-actin protein. **P < 0.01 vs. 21% O₂. B) β-Catenin and β-actin protein by Western Blotting for the recovery group and its corresponding control and densitometry of β-Catenin protein fold change, normalized to β-actin protein. *P < 0.05 vs. 21% O₂.

Figure 7.

A) Working Hypothesis showing Renin Angiotensin System (RAS) pathway and the effect of hyperoxia (by itself)) to upregulate TACE/ADAM17, which downregulates ACE-2 through the shedding of its ectodomain. ACE-2 shedding leads to an accumulation of the profibrotic angiotensin II (ANGII) and a decrease in the antifibrotic angiotensin 1-7 (ANG 1-7). Shaded areas are from earlier work (from 16, edited with permission). In the present study, Hypoxia preceding hyperoxia, in contrast, prevents TACE upregulation and/or downregulates TACE/ADAM17 and thus, presumably, prevents ACE-2 shedding. This allows the clearance of the profibrotic ANGI, increasing the antifibrotic ANG 1-7, and thus (at least theoretically) protects from fibrosis. AGT: angiotensinogen, ANG I: angiotensin I, ACE: angiotensin converting enzyme, TAPI-2: TNF-alpha Protease Inhibitor 2 (an inhibitor of TACE/ADAM17), AT-1: Angiotensin II receptor type 1, MAS: angiotensin 1-7 receptor.

(B) Experimental protocol from previous work (16), which showed that hyperoxia followed by normoxic recovery increases TACE/ADAM17 in IMR90 cells and thereby causes the shedding of ACE-2 ectodomain (see Panel A), which favors the profibrotic ANG II while simultaneously decreasing the antifibrotic ANG 1-7 (5,16).

(C) Hypoxia prior to hyperoxic exposure (followed by normoxic recovery) leads to a decrease in TACE, which would be expected to prevent ACE-2 from decreasing and in theory, protect against fibrosis.