THE EFFECT OF HYPOXIA ON ER-β EXPRESSION IN THE LUNG AND CULTURED PULMONARY ARTERY ENDOTHELIAL CELLS

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Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Master of Science
in the Program of Translational Science,
Indiana University

July 2013

Accepted by the Faculty of Indiana University, in partial
fulfillment of the requirements for the degree of Master of Science

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ACKNOWLEDGMENTS

I would like thank my mentor Dr. Tim Lahm for all his guidance, dedication and support. I am eternally grateful for the experience I have had in his lab and believe that any future success that I have is the result of his tutelage.

I also would like to extend special thanks and gratitude to Dr. Irina Petrache who has been an inspirational role model and to my advisory committee member Dr. Kelly Schweitzer for her direction and mentorship. Additionally, I would like to acknowledge all the Lahm lab and Petrache lab family who have been a joy to work with and to learn from especially Dr. Angelia Lockett.

I am most appreciative for the leadership of the Translational Science Master's program Dr. Mark Payne and his diligent program director Carrie Ann Hansel for their guidance and support through this journey. Additionally, I would like to thank Dr. Jack Buckley and the IU School of Medicine for helping me achieve this otherwise, unattainable dream.

I thank my family and friends for their support. Finally, I am most grateful and thankful for my fiancé David Kubick for his unconditional love and relentless support.

ABSTRACT

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THE EFFECT OF HYPOXIA ON ER-β EXPRESSION IN THE LUNG AND CULTURED PULMONARY ARTERY ENDOTHELIAL CELLS

17-β estradiol (E2) exerts protective effects in hypoxia-induced pulmonary hypertension (HPH) *via* endothelial cell estrogen receptor (ER)-dependent mechanisms. However, the effects of hypoxia on ER expression in the pulmonary-right ventricle (RV) axis remain unknown. Based on previous data suggesting a role of ER-β in mediating E2 protection, we hypothesized that hypoxia selectively up-regulates ER-β in the lung and pulmonary endothelial cells.

In our Male Sprague-Dawley rat model, chronic hypoxia exposure (10% FiO_2) resulted in a robust HPH phenotype associated with significant increases in ER- β but not ER-α protein in the lung *via* western blotting. More importantly, this hypoxia-induced ER-β increase was not replicated in the RV, left ventricle (LV) or in the liver. Hence, hypoxia-induced ER-β up-regulation appears to be lung-specific. *Ex vivo*, hypoxia exposure time-dependently up-regulated ER-β but not ER-α in cultured primary rat pulmonary artery endothelial cells (RPAECs) exposed to hypoxia (1% O_2) for 4, 24 or 72h. Furthermore, the hypoxia induced ER-β protein abundance, while not accompanied by increases in its own transcript, was associated with ER-β nuclear translocation, suggesting increase in activity as well as post-transcriptional up-regulation of ER-β.

Indeed, the requirement for ER-β activation was indicated in hypoxic ER-βKO mice where administration of E2 failed to inhibit hypoxia-induced pro-proliferative ERK1/2 signaling. Interestingly, HIF-1α accumulation was noted in lung tissue of hypoxic ER-βKO mice; consistent with previously reported negative feedback of ER-β on HIF-1α protein and transcriptional activation. In RAPECs, HIF-1 stabilization and overexpression did not replicate the effects of ER- β up-regulation seen in gas hypoxia; suggestive that HIF-1α is not sufficient for ER-β up- regulation. Similarly, HIF-1 inhibition with chetomin did not result in ER-β down-regulation. HIF-1α knockdown in RPAECs in hypoxic conditions is currently being investigated.

Hypoxia increases ER- β, but not ER-α in the lung and lung vascular cells. Interpreted in context of beneficial effects of E2 on hypoxic PA and RV remodeling, our data suggest a protective role for ER-β in HPH. The mechanisms by which hypoxia increases ER-β appears to be post-transcriptional and HIF-1α independent. Elucidating hypoxia-related ER-β signaling pathways in PAECs may reveal novel therapeutic targets in HPH.

Tim Lahm, MD (Chair)

TABLE OF CONTENTS

ABBREVIATIONS

18srRNA.....18s Ribosomal Ribonucleic Acid
ANOVA..... Analysis of the Variance
AF-1 ..... Activation Function-1
Akt.....Protein Kinase B
ARNT .....Aryl hydrocarbon Receptor Nuclear Translocator
APAH .....Associated Pulmonary Arterial Hypertension
CA-9.....Carbonic Anhydrase-9
cAMP..... Cyclic Adensoine Monophosphate
CBP/p300.....CREB Binding Protein/ E1A binding protein p300
$CoCl_2$ ..... Cobalt Chloride
CTM .....Chetomin
DMEM ..... Dulbecco's Modified Eagle Medium
DFO .....Deferoxamine
DMOG .....Dimethyloxaloylglycine
DMSO ..... Dimethylsulfoxide
DNA ..... Deoxyribonucleic Acid
DPN ..... Diarylpropionitrile
E2.....17beta-Estradiol
EGF..... Epidermal Growth Factor
Egr-1 .....Early Growth Response-1
EMT ..... Epithelial Mesenchymal Transition
eNOS ..... Endothelial Nitric Oxide synthase
ER ..... Estrogen Receptor
ERK1/2 .....Extracellular-Signal Regulated Kinases
ER-α..... Estrogen Receptor –alpha
ER-β..... Estrogen Receptor –beta
ESR1.....Estrogen Receptor 1 Gene
ESR2.....Estrogen Receptor 2 Gene
FiO_2.....Fraction of Inspired Oxygen
GAPDH ..... Glyceraldehyde 3-Phosphate Dehydrogenase
GPCRs.....G-Protein Coupled Receptors
HIF-1A..... Hypoxia Inducible Factor-1A Gene
HIF-1α .....Hypoxia Inducible Factor-1alpha
HIF-1β ..... Hypoxia Inducible Factor-1beta
HIF-2α .....Hypoxia Inducible Factor-2alpha
HIF-3α .....Hypoxia Inducible Factor-3alpha
HPAECs ..... Human Pulmonary Artery Endothelial Cells
HPF.....High Power Field
HPH ..... Hypoxic Pulmonary Hypertension
HRT.....Hormone Replacement Therapy
HRE .....Hypoxia Responsive Element
ICC.....Immunocytochemistry
IHC.....Immunohistochemistry
IGF-1..... Insulin-like Growth Factor-1
IPAH.....Idiopathic Pulmonary Arterial Hypertension
JNK .....c-Jun N-Terminal Kinases
KO.....Knockout
LV.....Left Ventricle
MAPKs .....Mitogen-Activated Protein Kinases

mER ..... Membrane Estrogen Receptor
MPI ..... Megapixel Intensity
NF-kB ..... Nuclear Factor Kappa B
NO ..... Nitric Oxide
P38 ..... p38 Mitogen-Activated Protein Kinase
PA ..... Pulmonary Artery
PAH ..... Pulmonary Arterial Hypertension
Patm ..... Atmospheric Pressure
PCR ..... Polymerase Chain Reaction
P-ERK ..... Phosphorylated Extracellular Signal Regulated Kinase
PI3K ..... Phosphatidylinositol 3-kinase
PHD ..... Prolyl Hydroxylase
PPARs ..... Peroxisome Proliferator-Activated Receptors
RISC-free ..... RNA-Induced Silencing Complex
RNA ..... Ribonucleic Acid
RPAECs ..... Rat Pulmonary Artery Endothelial Cells
RPASMCs ..... Rat Pulmonary Artery Smooth Muscle Cells
RV ..... Right Ventricle
SiRNA ..... Small Interfering Ribonucleic Acid HIF-2α
SMA ..... Smooth Muscle Actin
Src ..... Tyrosine-Protein Kinase Src
vWF ..... Von Willebrand Factor
WT ..... Wild Type

I. INTRODUCTION

A. Hypoxic Pulmonary Hypertension: Pulmonary hypertension is a syndrome that embraces heterogeneous clinical entities sharing the common hemodynamic features of elevated pulmonary arterial and right ventricular pressures culminating in right heart failure. Hypoxic Pulmonary Hypertension (HPH) is a grave consequence of all entities that result in low alveolar oxygen tension [1, 2]. It is characterized by pulmonary vascular bed remodeling culminating in right ventricular (RV) failure and death [3, 4]. Despite its frequency compared to other types of PH [1]; no specific treatment for HPH exists. Moreover, current therapeutics worsen ventilation-perfusion mismatch and consequently, oxygenation in HPH [5-7].

B. The Estrogen Paradox in Pulmonary Hypertension: Female preponderance is a known epidemiological feature of Pulmonary Arterial Hypertension (PAH). Review of the French registry and REVEAL registry reported female to male ratios in PAH of 1.9:1 and 4.3:1 respectively [8, 9]. REVEAL demonstrates a 4.1:1 female-to-male ratio among patients with Idiopathic PAH (IPAH), and a 3.8:1 ratio among those with Associated PAH (APAH). Consistent with observations from the NIH registry, REVEAL also shows a higher female preponderance at 5.4:1 ratio among blacks. This is probably the sum of female preponderance in the majority of subclasses of PAH. These subclasses include idiopathic and heritable PAH as well as PAH associated with connective tissue diseases, drugs and portopulmonary hypertension. Despite no clear reason for this disparity in female predominance among registries, some speculate that the increase in use of hormone replacement therapy (HRT) between NIH and REVEAL registries could be responsible [10].

Despite the female predominance in PAH, female gender is associated with improved survival, [11-13], termed Estrogen Paradox [14]. The main culprit to explain female prevalence in PAH naturally has been sex hormones. Negative effects of estrogen and potentially favorable effect of androgens on pulmonary vasculature is a rather logical and attractive simple explanation. Other theories include female preponderance in autoimmune disorders and connective tissue diseases, alloimmunization associated with pregnancy and childbirth as well as environmental exposures including estrogen containing medications and anorexigens [15]. More recently, human and animal data

has suggested an association between PAH and metabolic syndrome that further highlights sex hormones and gender differences [16].

C. **Estrogen in HPH; Animal and Human Data:** In HPH, 17β- Estradiol (E2) has been shown to be protective in both animal and human data. Female sex confers a protective phenotype in high altitude-related HPH [17, 18]. Moreover, estrogen surge in pregnancy attenuates hypoxic pulmonary vasoconstriction [19, 20]. HRT is also thought to be a predictor of improved RV function; both in postmenopausal women [21] and PAH patients with scleroderma [22, 23]. A recent study interrogated the REVEAL database for differences between male and female PAH patients [24], and found no differences in exercise capacity, or diagnostic/treatment strategies. Even though men had statistically significant higher mean pulmonary artery and right atrial pressure, the clinical significance of these subtle differences is questionable. Not surprisingly, more women had CTD-PAH and CHD-PAH, while more men had portopulmonary hypertension and HIV-associated PAH. Thyroid disease and depression were more common in women. Studies suggest that healthy women as well as women with PAH have a higher right ventricular ejection fraction than men (assessed by cardiac magnetic resonance imaging) [25-27] providing a potential explanation for the observed gender differences in outcomes. A recent study identified provided another potential explanation by demonstrating that women exhibit a better response to treatment with endothelin receptor antagonists [28].

In animal studies, exogenous and endogenous E2 attenuated isolated pulmonary artery (PA) ring vasoreactivity; effects increased with the phasic increase of E2 during menstrual cycle [29, 30]. Additionally, specific ER agonists resulted in NO-mediated vasorelaxation in isolated PA rings [31]. In high altitude and its simulators i.e. hypobaric hypoxia, female gender confers protection from HPH in rodent and swine models as compared to male counterparts [32-34]. Finally, ovariectomy exacerbates HPH, whereas E2 replacement in ovariectomized animals attenuates the disease [35, 36]. Recently, E2 was found to effectively attenuate PH phenotype in hypoxic rat model of PH [37]. E2 was also found to rescue preexisting PH in a monocrotaline- induced PH rat model [38]. E2 appears to exert its protective effects *via* endothelium-centered and estrogen receptor (ER) dependent effects [31, 37].

D. Estrogen Receptors Physiology: Estrogen receptors are proteins that belong to the nuclear-receptor superfamily of transcription factors [39, 40]. ERs are products of different genes on different chromosomes [41, 42]. These receptors share significant homology in their DNA and ligand- binding domains, less so in their N-terminus (ligand-independent activation function (AF-1) region) [43]. Isoforms of each ER exist due to alternative splicing [44] and can exist in hetero or homodimers [45, 46]. ER genes are abundantly expressed in the histologically normal lung; ER-β being more abundant than ER-α [47-50]. These receptors are believed to have distinct tissue expression patterns as well as biological functions [42, 49, 50]. ER subtypes - α and β- traditionally exert their effects *via* genomic modulation [43, 51]. In the late 1970's, Membrane-bound Estrogen Receptors (mERs) were first described to trigger rapid generation of cAMP [52, 53]. Since then, it has become known that these receptors exert non-genomic effects *via* second messenger signaling cascades including nitric oxide (NO), calcium influx, G-protein–coupled receptors (GPCRs), protein kinases including phosphoinositol-3 kinase (PI3K), serine-threonine kinase Akt receptor and mitogen-activated protein kinase (MAPK) family members (ERK/ JNK /p38) as well as tyrosine kinases including epidermal growth factor (EGF) receptor and insulin-like growth factor (IGF)-1 receptor, nonreceptor tyrosine kinase Src, and protein kinases A and C [43, 54-56]. **Figure 1** represents a schematic of genomic and non-genomic ER signaling.

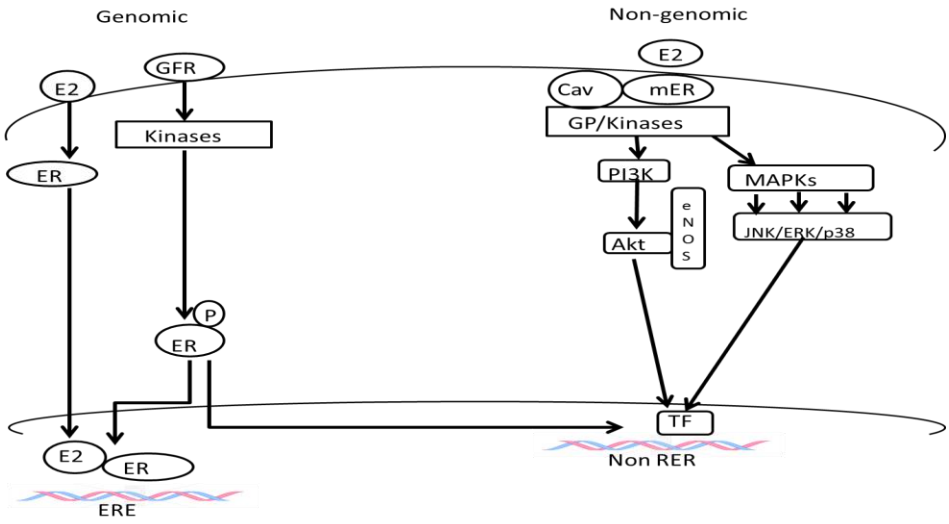

Figure 1. Representative schematic of genomic and non-genomic ER signaling.

In the context of PH, E2 is known to be a rapid activator of endothelial nitric oxide synthase (eNOS) *via* phosphatidylinositol 3 (PI3)-Kinase- Akt pathway [57]. Additionally, E2 is also known to up-regulate NOS gene transcription [58]. Moreover, E2 is thought to enhance eNOS function *via* the enactment of plethora of cellular effects including inhibition of cytokine-induced downregulation of NOS III gene expression, posttranslational modification of NOS III protein, increased cofactor or L-arginine availability, alongside modulation of NO degrading systems [59].

ERK1/2 -a member of the MAPK family- is thought to account for the antiproliferative effects of E2. In a HPH rat model, E2 *via* ER-dependent fashion was shown to attenuate hypoxia- induced ERK1/2 activation in the lung and RV [37]. The anti-inflammatory effects of E2 in the lung in a rat trauma-hemorrhage model were also shown to be mediated *via* an ERK1/2 pathway [60]. At the genomic level, E2 was shown to attenuate hypoxia-induced endothelin-1 gene expression. This attenuation is thought to be due to decreased Hypoxia Inducible Factor-1 (HIF-1) activity possibly through competition for limiting quantities of CBP/p300 [35].

Regulation of cellular ER concentration is a key component governing cellular responsiveness to ER agonists, antagonists and modulators. For ER-α, the primary regulator of its stability is the ligand itself, E2 [61]. In the absence of E2, the half-life of ER is about 5 days, but only 3-4 hours in the presence of E2 [62]. In human breast cancer cell lines, E2 is thought to induce decline in both ER-α protein and mRNA [63]. In eukaryotic cells, the ubiquitin-proteasome pathway is the major mechanism for the targeted protein degradation of proteins with short half-lives [64, 65]. Protein ubiquitination involves covalent attachment of ubiquitin to lysine residues of proteins targeted for degradation followed by formation of polyubiquitin chains [62]. Ubiquitinated ERs are then recognized and degraded by the multi-subunit protease complex, the 26S proteasome. Interestingly, ubiquitination of the members of the nuclear hormone receptor superfamily is believed to modulate their transcriptional activation [66]. Moreover, proteasome-dependent degradation of steroid receptors requires transcriptional activity; suggesting that degradation and receptor transactivation are mutually interdependent [67]. Evidence in uterine artery endothelial cells exists to

support the presence of differential regulation of proteasome dependent turnover for ER-α and ER-β [61].

E. Crosstalk between Hypoxia Inducible Factor and Estrogen Receptor: HIF-1 is the master regulator of the cellular oxygen homeostasis. In the lung, the crosstalk between hypoxia responsive pathways and nuclear receptors including ER is not entirely understood. Hypoxia enhances or decreases transcriptional activation of nuclear receptors including ERs depending on the experimental setting [68-72]. In human breast cancer cells, HIF-1 induced downregulation of ER-α that is mediated *via* ubiquitin-proteasome pathway degradation [69]; an effect believed to be facilitated specifically *via* ARNT subunit (HIF-1β) [73, 74]. Moreover, in HEK293 cells, hypoxia activated ligand-independent transcriptional activation of ER-α, possibly takes place through the interaction between HIF-1α and ER-α. We theorized that hypoxia will selectively up-regulate ER-β in the lung and PA endothelial cells and that this up-regulation is likely HIF-1α dependent. Review of the crosstalk between HIF-1 and ER-β is discussed at length in section IV. **Figure 2** depicts normoxic and hypoxic HIF regulation.

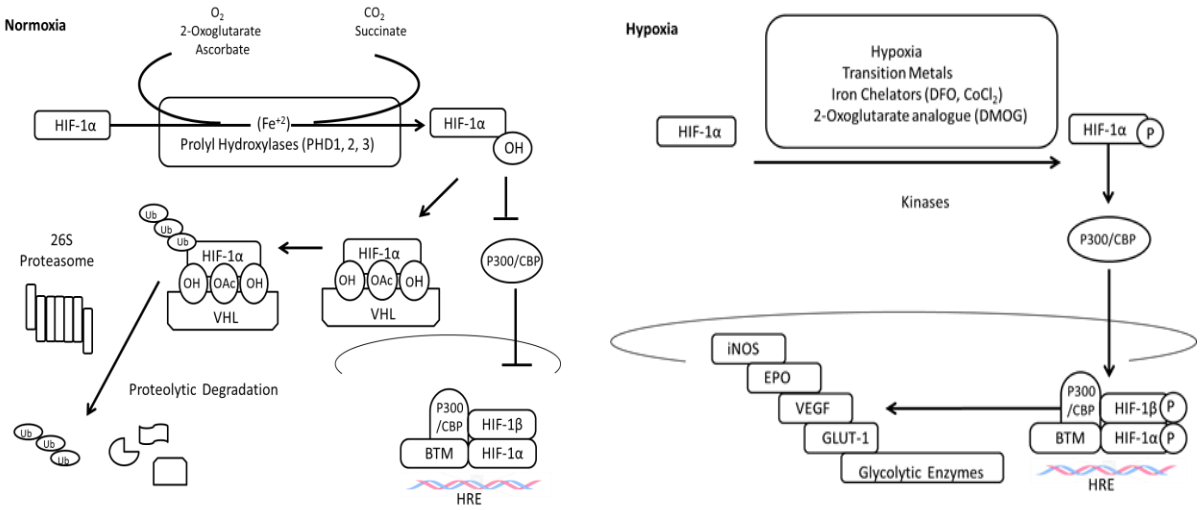

Figure 2. Representative schematics of HIF-1α degradation pathway in normoxia (left panel) and activation pathway in hypoxia (right panel).

II. MATERIALS AND METHODS

Animal Experiments and *in vivo* Hypoxia

Male Sprague-Dawley rats (250-275 g; Charles River) were exposed to 2 weeks of hypobaric hypoxia (P_{atm}= 362 mmHg; equivalent to 10% Fi_{O_2} at sea level) as described previously [37]. In a second experiment, male ER-β knockout mice (on C57BL/6 background; 20-22 g; Jackson Laboratories) were exposed to 3 weeks of hypobaric hypoxia (P_{atm}= 362 mmHg; equivalent to 10% Fi_{O_2} at sea level). Rats or mice were treated with E2 (75 μg/kg/d *via* subcutaneous pellets) for the entire duration of hypoxia exposure. At the end of the 2 or 3 week hypoxia exposure, animals were sacrificed and their lungs and RV harvested for analysis. All animals received care in compliance with the Guide for the Care and Use of Laboratory Animals. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine.

Cell Culture and Reagents

Primary rat PAECs were provided by Drs. Troy Stevens and Diego Alvarez (University of South Alabama Tissue and Cell Culture Core). Cells were derived from male Sprague-Dawley rats (350-400 g) and maintained up to passage 15 in Dulbecco's modified Eagle high-glucose medium (Sigma-Aldrich; St. Louis, MO) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37 °C in 5% CO_2 and 95% air. RPAEC phenotype was confirmed by cell morphology and by Western blotting and immunofluorescence staining for von Willebrand factor (1/400; Abcam-6994; Cambridge, MA). Cells were used for experimentation at 70% confluence. Similar experiments were done with human PAECs (Lonza; Hopkinton, MA) and rat PA smooth muscle cells (PASMCs; from T. Stevens and D. Alvarez, USAB).

In Vitro Hypoxia

Hypoxia exposure (4, 24 and 72 hours) occurred in a dedicated workstation (Ruskinn, Inc., Pencoed, UK). Oxygen concentration in the chamber was measured in real time and constantly adjusted to maintain the desired concentration. Control cells of identical

passage and confluence were grown concomitantly in a regular incubator at 21% O_2.

Chemical Hypoxia and HIF Inhibition

Normoxic HIF-1α activation ("chemical hypoxia") was induced with the prolyl hydroxylase inhibitor deferoxamine (DFO; 10 or 100 μM) (Sigma-Aldrich; St. Louis, MO). RPAECs were exposed to room air and treated with DFO for 4, 16, 24 or 48 hours. Control cells were either untreated or treated with vehicle (H_2O). HIF-1α activation was inhibited with the known HIF-1α inhibitor chetomin (5, 25, 50 or 100nM) (Sigma-Aldrich; St. Louis, MO)). RPAECs were pre-treated with chetomin for 4 hours in 21% O_2 and then transferred to 1% O_2 for 24 hours. Control cells of identical confluence and passage were grown concomitantly at 1% O_2 and were either untreated or treated with chetomin vehicle (dimethylsulfoxide (DMSO)) (Sigma-Aldrich; St. Louis, MO).

Immunocytochemistry (ICC)

Cells were grown on coverslips and exposed to room air (21% O_2) or hypoxia (1% O_2) for 4, 24 or 72 hours. Cells were fixed with paraformaldehyde, blocked with goat serum and then stained with rabbit polyclonal IgG against ER-α and ER-β antibodies (1/200; Santa Cruz HC-20 and HC-150, respectively; Dallas, TX). Secondary flurochrome-conjugated anti-rabbit antibody from was used (1/200; Alexa Fluor 488). DAPI (Invitrogen; Valencia, CA) mounting media was used for nuclear staining. Experiments were run in duplicates. Images were taken with fluorescence microscopy. ER-α and ER-β expression was quantified as average megapixel intensity normalized for number of nuclei per HPF by Image J (NIH) in a blinded fashion.

Immunohistochemistry (IHC)

Lung sections (4 mm) from normoxic and hypoxic rats were stained for ER-α or ER-β (1/10: Dako PPG 5/1; Carpinteria, CA) and examined by light microscopy (340 objective). The degree of ER-β expression was assessed in a blinded fashion.

Quantification of ER-β expression was done by Metamorph Software (Center Valley, PA).

Western Blotting

The following antibodies were used for western blotting: ER-α (1/50; Santa Cruz HC-20; Dallas, TX), ER-β (1/200; Santa Cruz HC-150; Dallas, TX), HIF-1α (1/500; Novus Biological, Littleton, CO), Phospho-ERK1/2 and total ERK1/2 (1/1000; Cell Signaling, Boston, MA). 50 ug of protein was used for ER-α and HIF-1α quantification, 13 ug of protein for ER-β and 12 ug for ERK1/2 and Phospho ERK1/2 quantification. Cells were harvested in either RIPA buffer (Sigma- Aldrich; St. Louis, MO) or Cell Lysis Buffer (Cell Signaling; Boston, MA) for ER-α/ER-β and HIF-1α analyses, respectively. Vinculin was used as internal control (1/2000; Calbiochem, Billerica, MA). Densitometry was performed *via* Image J (NIH).

Real Time-Polymerase Chain Reaction (RT-qPCR)

Total RNA was isolated from RPAECs or rat lung or RV homogenates using the RNeasy Plus Mini Kit (Qiagen; Valencia, CA). QuantiTect Reverse Transcription Kit (Qiagen; Valencia, CA) was used to make cDNA from 2 µg of total RNA. The reaction volume for qPCR was 25 µl [12.5 µl SYBR Green Master Mix (Qiagen; Valencia, CA), 1 µl primer, 2 µl cDNA and 9.5 µl RNAse free water (Qiagen; Valencia, CA). PCR parameters are as follows: *stage 1*, 50°C for 2 min; *stage 2*, 95°C for 10 min; *stage 3*, *step 1* = 95°C for 15 s for 45 cycles, *step 2* = 60°C for 1 min (data collection); *stage 3*, *step 1* = 95°C for 15 s, *step 2* = 60°C for 15 s, *step 3* = 95°C for 15 s; (dissociation curve) *stage 4*, −72°C hold. 50 ng of cDNA was used per qPCR reaction; DNA amplification was monitored by incorporation of SYBR green dye. Rat primers for ER-α (ESR1), ER-β (ESR2), HIF-1α (HIF1A) as well as GAPDH and 18SrRNA were purchased from (SABioscheinces; Frederick, MD). Data was analyzed using ΔCT method.

Transfection Experiments

Rat PAECs were grown in full media until 70-90% confluence and transfected with human HIF-1α (HA-HIF1α P402A/P564A-pcDNA3) (Addgene; Cambridge, MA; provided by Dr. Mircea Ivan) in separate experiments. HIF-1α control plasmid (pcDNA3) was used at equal doses of 2.5-5.0 ng. Transfection was done in antibiotic free media with Lipofectamine® LTX & Plus™ Reagent in Opti-MEM (Invitrogen; Valencia, CA).

Statistical Analyses

Statistical analyses were performed with GraphPad Prism 6 (La Jolla, CA). The differences between groups were compared using unpaired Student t-test (for comparison of 2 groups) or ANOVA (for comparison of > 2 groups). All cell experiments were done at least three times and data were expressed as mean ±SEM. Animal experiments were expressed as mean± SEM. Statistically significant difference was accepted as $p < 0.05$.

III. RESULTS

A. Hypoxia increases ER-β but not ER-α in rat lung. Evidence exists to support a unique role of ER-β in HPH. ER-β blockade attenuates E2's effects during hypoxia *in vivo* and in RPAECs [37]. Additionally, selective activation of ER-β attenuates hypoxic vasocontraction in isolated PA rings in an endothelium-dependent manner [31]. Moreover, selective ER-β blockade resulted in loss of E2 mediated protection in a monocrotaline PH model [38]. We hypothesized that hypoxia would selectively up-regulate ER-β but not ER-α in the lung. We investigated the effect of hypoxia on ERs *in vivo* using a two-week Sprague Dawley male rats' hypoxia model. As hypoxia culminates in right ventricular failure; ER expression in the RV was also studied under hypoxic conditions. In the rat lung, ER-β protein was increased with western blot **(Figure 3A- B)**. Moreover, IHC revealed a trend towards increased ER-β staining in hypoxic rat lung sections **(Figure 3C)**. This ER-β up-regulation was not associated with increased ER-β mRNA in the lung **(Figure 3D)**. Interestingly, hypoxia failed to up-regulate ER-β in hypoxic rat homogenates of RV, LV and liver **(Figure 4A-D)**. Additionally, hypoxia did not increase ER-α neither in the lung nor in the RV **(Figure 5A-D)**. In conclusion, hypoxia- induced ER up-regulation appears to be ER-β as well as lung specific.

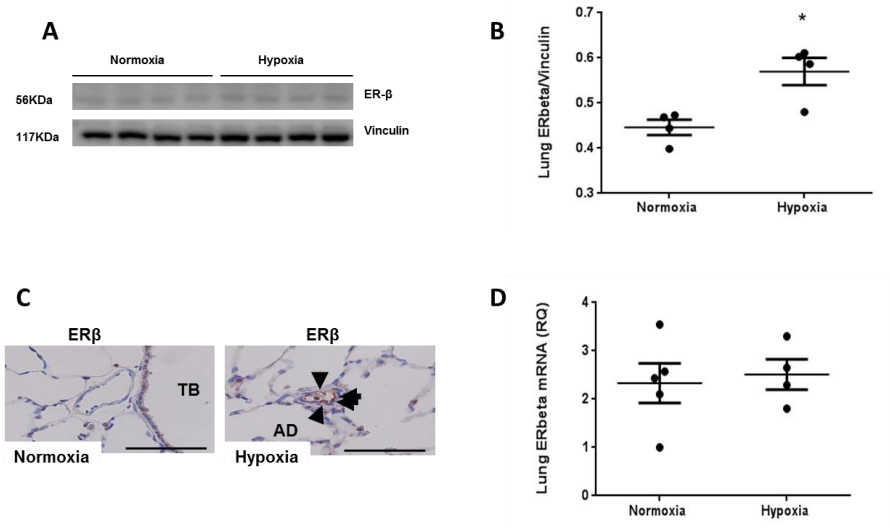

Figure 3. Hypoxia increases ER-β expression in the lung. A. ER-β protein expression in rat lung homogenates after 2 weeks of hypoxia versus normoxia using western blot. **B.** Expression levels of lung ER-β quantified by densitometry **C.**

Representative IHC images of lung sections are shown; note the increase in ER-β-positive cells (arrows) in hypoxic PAs at the level of terminal bronchioles (TB) or alveolar ducts (AD). Positive staining for ER-β was mainly present in PA endothelial cells, while there was no significant staining of PA smooth muscle cells. **D.** ER-β gene expression in the lung measured by RT-qPCR. Size bars =50 μm. *p < 0.05.

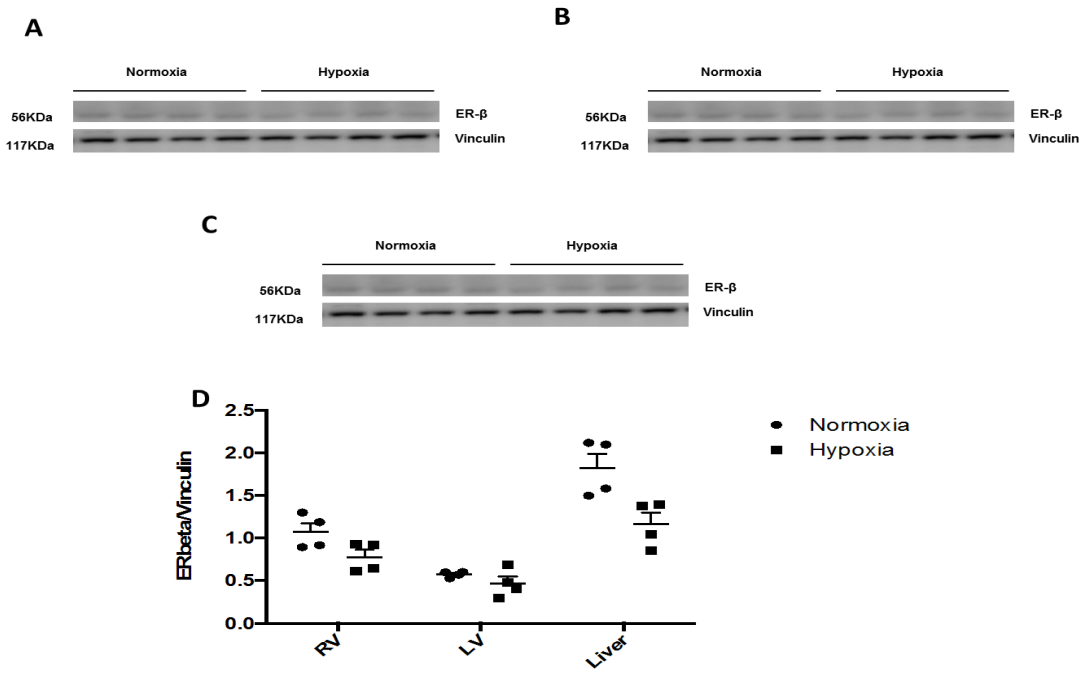

Figure 4. **Hypoxia-induced increase in ER-β is specific to the lung. A-C.** ER-β protein expression in rat homogenates of RV, LV and liver, respectively, after 2 weeks of normoxia (●) versus hypoxia (■) using western blot. **D.** Expression levels of RV, LV and liver ER-β quantified by densitometry at normoxic and hypoxic conditions.

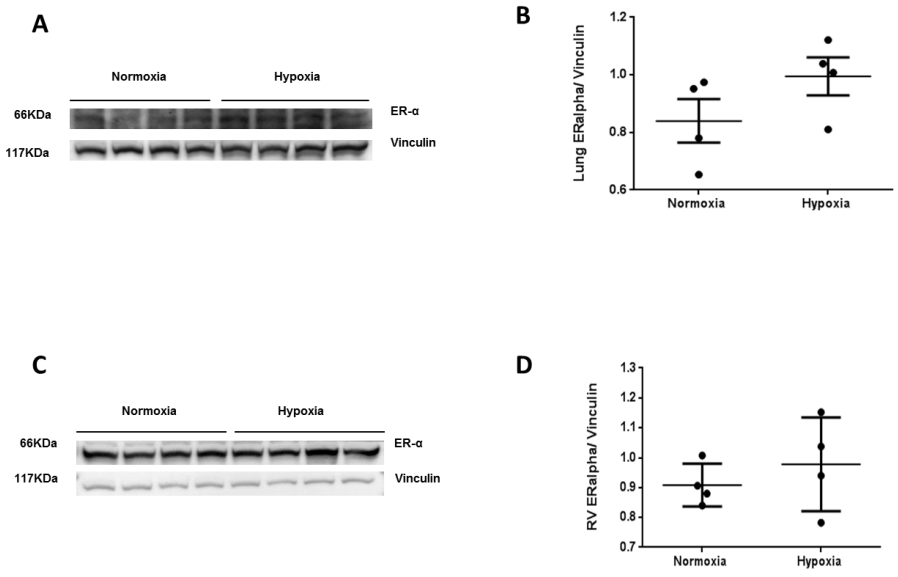

Figure 5. Hypoxia does not increase ER-α in the lung or in the right ventricle. A. ER-α protein expression in rat lung homogenates after 2 weeks of hypoxia versus normoxia using western blot. **B.** Expression levels of lung ER-α quantified by densitometry. **C.** ER-α protein expression in rat RV homogenates after 2 weeks of hypoxia versus normoxia. **D.** Expression levels of RV ER-α quantified by densitometry.

B. Hypoxia increases ER-β but not ER-α in rat PAECs. Previous work from our lab has shown that E2 appears to exert its protective effects *via* endothelium-centered and estrogen receptor (ER) dependent effects [31, 37]. Given our *in vivo* data, we decided to further investigate the effects of hypoxia *in vitro* using cultured primary rats PAECs. Interestingly, hypoxia increased ER-β protein time-dependently at 4, 24 and 72 hours of hypoxia as quantified by ICC **(Figure 6A-B)** as well as western blot **(Figure 6C-D)**. This increase was associated with a pattern of nuclear translocation evident on ICC suggestive of ER-β activation. Parallel experiments to investigate the effect of hypoxia on ER-α did not reveal a significant increase in ER-α protein *via* ICC **(Figure 7A-B)** and western blot **(Figure 7C-D)**. In conclusion, hypoxia-induced ER-β up-regulation is replicated *in vitro* as hypoxia time-dependently increased ER-β but not ER-α in hypoxic RPAECs.

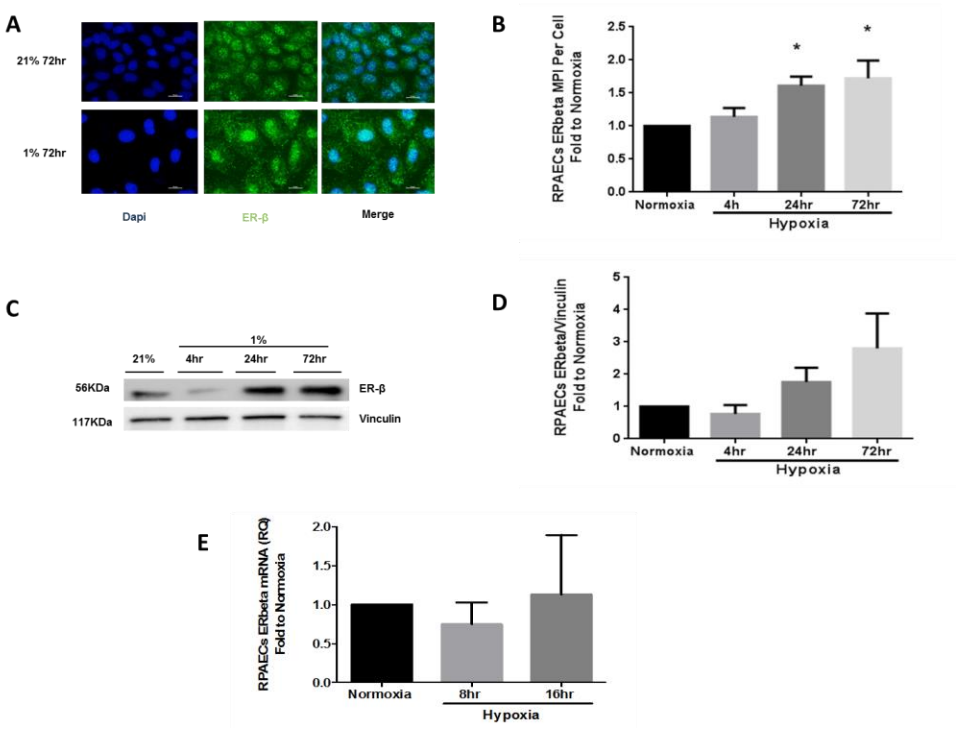

Figure 6. Hypoxia increases ER-β expression in cultured rat pulmonary artery endothelial cells (RPAECs). A. Representative ICC images of RPAECs at 72 hours treated with hypoxia versus normoxia. ER-β is stained in green. Note that ER-β staining suggested increased nuclear translocation. **B.** ER-β expression levels were quantified by normalizing megapixel intensity by the number of cells (nuclei stained with DAPI) with Image J. ER- β expression levels expressed as 4, 24 and 72 hours hypoxia fold change compared to normoxia **C.** ER-β protein expression *via* western blot in RPAECs after 4, 24 and 72 hours of hypoxia versus normoxia. **D.** Expression levels of ER-β quantified by densitometry. **E.** ER-β gene expression in RPAECs measured by RT-qPCR. Size bars = 10 μm. Data represents the mean ± SEM (n=3). *$p < 0.05$.

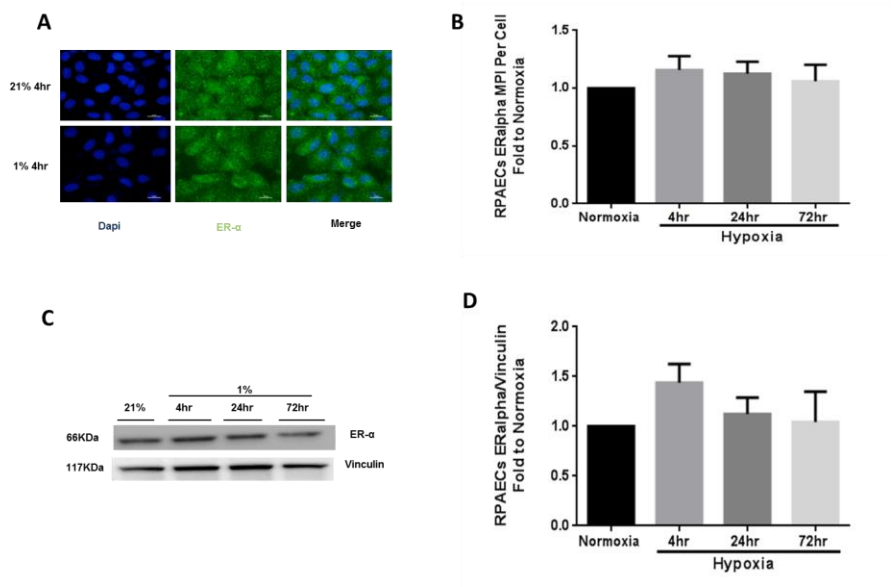

Figure 7. Hypoxia does not increase ER-α expression in cultured rat pulmonary artery endothelial cells (RPAECs). A. Representative ICC images of RPAECs at 4 hours treated with hypoxia versus normoxia. ER-β is stained in green. **B.** ER- α expression levels were quantified by normalizing megapixel intensity by the number of cells (nuclei stained with DAPI) with Image J. ER-α protein expression levels expressed as 4, 24 and 72 hours hypoxia as fold change compared to normoxia. **C.** ER-α protein expression *via* western blot in RPAECs after 4, 24 and 72 hours of hypoxia versus normoxia. **D.** Expression levels of ER- α quantified by densitometry. Size bars = 10 μm. Data represents the mean ± SEM (n=3).

C. Hypoxia increases ER-β in the lung and RPAECs post-transcriptionally. Potential hypoxia-responsive element (HRE) sequences exist in the human, rat and mouse ESR2 gene promoter region (Blast and Ensemble search). However, the functional significance of the putative HREs remains to be determined. To investigate whether hypoxia- mediated ER-β overexpression is due to transcriptional activation, ER-β mRNA RT-qPCR was performed both in the hypoxic lung and RPAECs. Interestingly, ER-β up-regulation was not preceded nor paralleled by a similar change in ER-β message *in vivo* **(Figure 3D)** nor in RPAECs at 8-16 hours **(Figure 6E)**. These results are suggestive of a post-transcriptional mechanism of ER-β up-regulation.

D. Normoxic HIF-1α stabilization is not sufficient to increase ER-β in RPAECs. Hypoxia- inducible factor-1 is the master regulator of O_2 homeostasis as well as the main transcription factor mediating molecular hypoxic responses. Despite no evidence to suggest hypoxia induced up-regulation of ER-β mRNA, other mechanisms of HIF-1α induced ER-β up-regulation could not be excluded. We hypothesized that hypoxia mediates ER-β overexpression *via* HIF-1. To test this hypothesis, chemical hypoxia with HIF-1 simulators like deferoxamine (DFO) was induced and its effect on ER-β expression studied under normoxic conditions in RPAECs. Notably, DFO and other chemicals that stabilize HIF-1 (like DMOG and $CoCl_2$) lack specificity and likely stabilize other members of the HIF family like HIF-2 and HIF-3. However, our data revealed that DFO increases HIF-1 protein (*via* iron chelation and inhibition of prolyl hydroxylases (PHD)-dependent HIF-1 degradation) in a dose-dependent fashion **(Figure 8A-B).** This HIF-1α increase was not paralleled with an increase in ER-β at 24 hours **(Figure 8C-D)**, concluding that normoxic HIF-1α stabilization is not sufficient for ER-β overexpression.

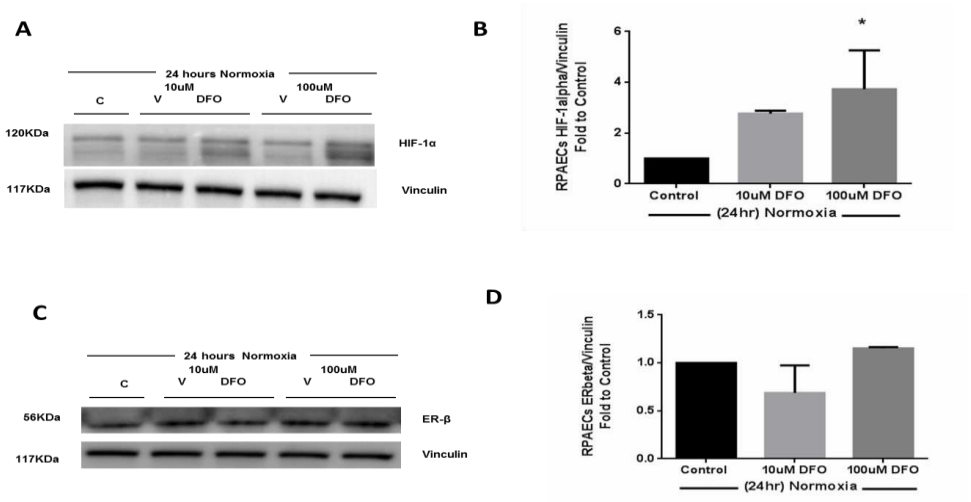

Figure 8. Chemical Hypoxia with DFO does not increase ER-β expression in cultured rat pulmonary artery endothelial cells (RPAECs). Effect of DFO treatment (10 or 100 μM; 24 hours normoxia) or vehicle (H_2O) on HIF-1α **(A)** and ER-β **(C)** protein expression *via* western blot, using vinculin as loading control. Protein expression was quantified with densitometry **(B, D)** and is expressed as fold change compared to control. Data represents the mean ± SEM (n=3). *$p < 0.05$.

E. HIF-1α is not necessary for hypoxia-induced ER-β overexpression in RPAECs. To fully understand the potential relationship between HIF-1α and hypoxia-mediated ER-β overexpression, HIF-1 inhibition with chetomin (CTM) was pursued. CTM is a HIF inhibitor that blocks the interaction of HIF-1α and HIF-2α with transcriptional co-activators p300 and CBP. Consequently, CTM inhibits HIF-1 transcriptional activation (and its downstream signaling like carbonic anhydrase-9 (CA-9). Although CTM is known to decrease HIF-1α protein, it has been reported to have the opposite effect in certain cell lines [75]. HIF-1 inhibition with CTM decreased HIF-1α in a dose dependent fashion **(Figure 9A)**. However, HIF-1 inhibition with CTM failed to attenuate hypoxia induced ER-β up-regulation in RPAECs **(Figure 9B-C)** suggesting that HIF-1α is not necessary for hypoxia-induced ER-β overexpression.

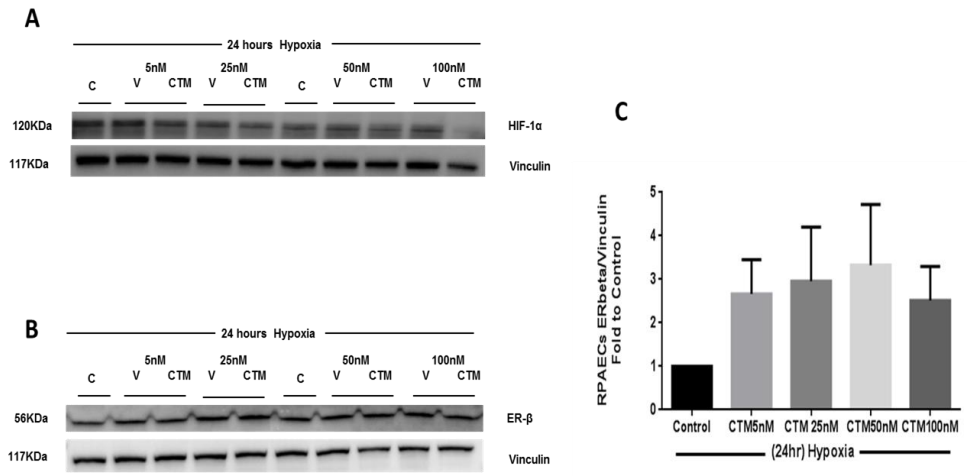

Figure 9. HIF inhibition with chetomin does not decrease ER-β expression in cultured rat pulmonary artery endothelial cells (RPAECs). Effect of CTM treatment (5, 25, 50 and 100 nM; 4 hours pre-incubation; 24 hours hypoxia) or vehicle (DMSO) on HIF-1α **(A)** and ER-β **(B)** protein expression *via* western blot, using vinculin as loading control. ER-β protein expression was quantified with densitometry **(C)** and is expressed as fold change compared to control. Data represents the mean ± SEM (n=3).

F. Normoxic HIF-1α overexpression is not sufficient to increase ER-β in RPAECs. To isolate the effect of HIF-1 from other members of the HIF family; HIF-1α was exogenously overexpressed in RPAECs. In normoxic conditions, despite an increase in HIF-1α in RPAECs following transfection **(Figure 10A-B)**, ER-β expression levels were unchanged **(Figure 10C-D)** suggesting that normoxic HIF-1α overexpression is not sufficient to increase ER-β in RPAECs.

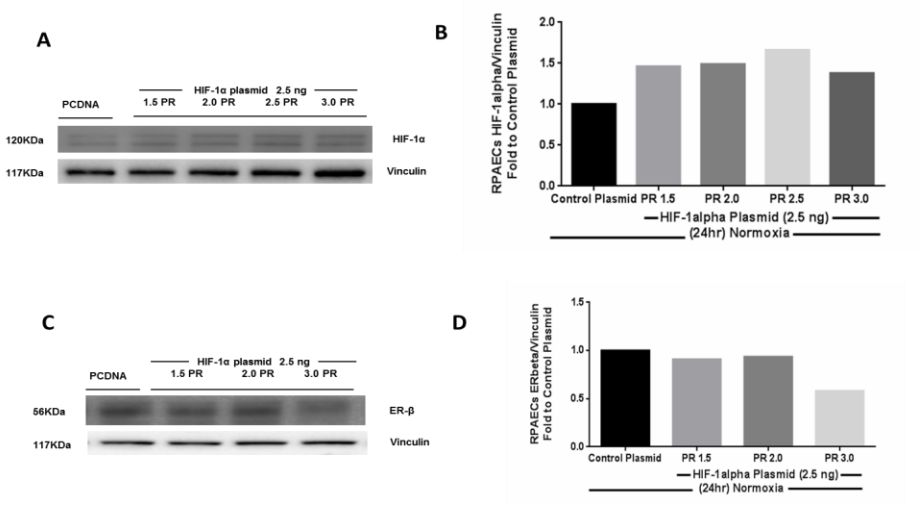

Figure 10. HIF-1α overexpression does not increase ER-β expression in cultured rat pulmonary artery endothelial cells (RPAECs). A. ER-β protein expression in RPAECs treated with control conditions, control plasmid (pcDNA 3.1) and HIF-1α plasmid at 2.5 ng; 24 hours normoxia. **B.** Expression levels of ER-β quantified by densitometry as fold change compared to control plasmid. Data represent (n=1).

G. ER-β mediates attenuation of pulmonary vascular remodeling and pro-proliferative signaling. With a plethora of evidence suggesting that ER-β mediates protection in HPH [31, 37], loss of protective ER-β mediated effects was explored in a 3-week model of HPH in ER-β knockout mice in the presence of E2. Lung sections from four groups of hypoxic animals were used; wild type (WT), WT with E2 treatment, ESR1 knockout (KO) mice with E2 treatment and finally ESR2 KO mice with E2 treatment. IHC for SMA was done to evaluate small- and medium-sized pulmonary

arterial bed thickness as a function of hypoxia-induced vascular remodeling. Interestingly, ESR2KO+E2 mice displayed vascular remodeling approximating that of the WT mice and significantly increased compared to the two other groups **(Figure 11A-B)**. These findings suggest that the loss of ER-β is associated with worse vascular remodeling that is not rescued with E2. Additionally, proliferative signaling in lung homogenates from the same groups of animals was evaluated with western blot for total ERK1/2 and P-ERK1/2. ESR2KO+E2 mice showed a level of ERK1/2 activation similar to the WT mice and increased compared to the other two groups **(Figure 12A-B)**, despite not reaching statistical significance. This is suggestive of the role of ER-β in attenuating pro-proliferative signaling in the hypoxic lung

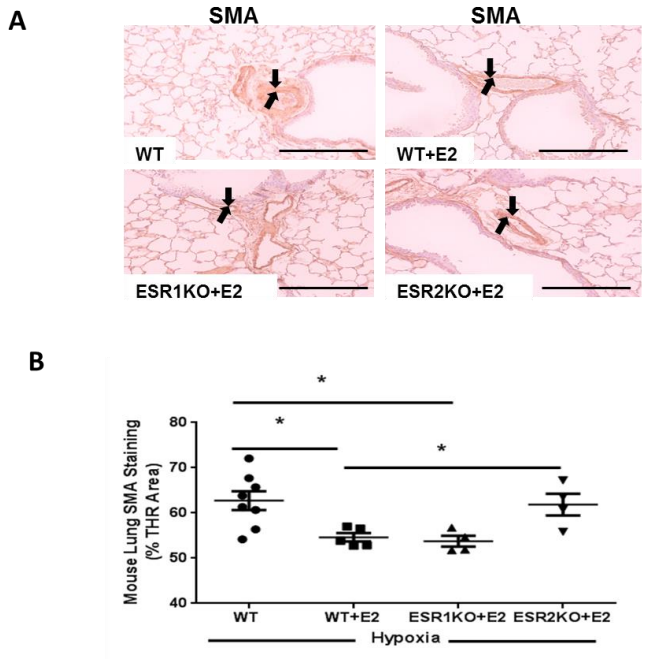

Figure 11. ESR2KO mice display increased pulmonary vascular remodeling in the hypoxic lung despite E2 treatment. A. Representative IHC images of lung sections are shown from WT, WT+E2, ESR1 KO+E2, ESR2 KO+E2 mice exposed to 3 weeks of hypoxia. Note the increase in vascular wall thickening in WT and ESR2KO+E2 mice compared to WT+E2 and ESR1KO+E2 represented by staining for SMA in walls of small- and medium-sized pulmonary arteries (arrows). **B.** Quantification of SMA staining *via* Metamorph. Size bars = 50 μm. *$p < 0.05$.

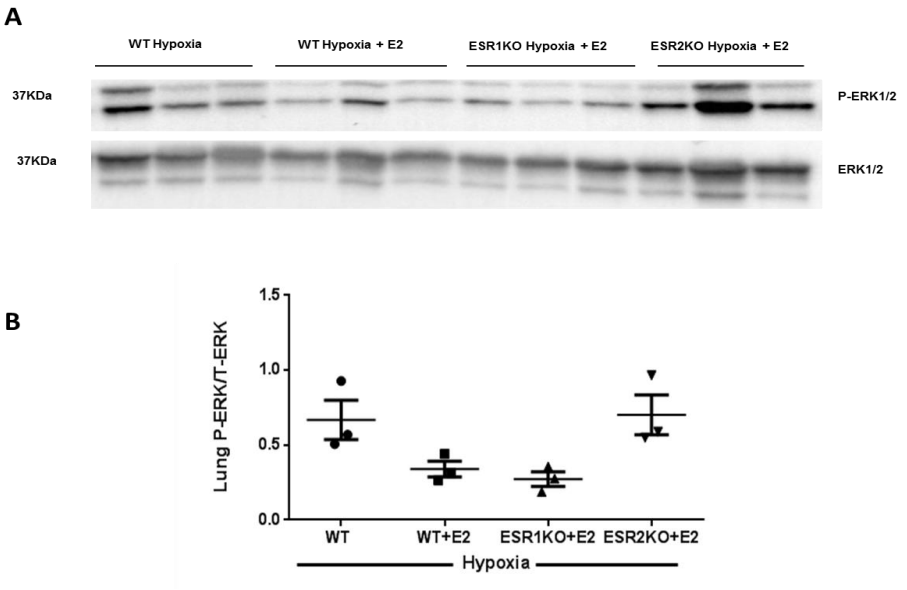

Figure 12. ESR2 knockout mice display increased ERK1/2 activation in the hypoxic lung despite E2 treatment. A. P-ERK1/2 and total ERK1/2 protein expression in mouse lung homogenates from WT, WT+E2, ESR1 KO+E2, ESR2 KO+E2 mice exposed to 3 weeks of hypoxia. **B.** Expression levels of lung P- ERK/ERK quantified by densitometry.

H. ER-β Knockout mice exhibit higher HIF-1α levels in the lung. The cross talk between hypoxia-responsive pathways and ERs has been extensively studied in the oncology literature. A body of evidence exists to support the notion that ER-β mediates HIF-1 inhibition mainly *via* regulation of HIF-1 degradation. In the same model of HPH, HIF-1α was measured in lung homogenates from the four groups of animals. Interestingly, HIF-1α expression was increased in ESR2KO+E2 mice- distinctly from the rest of the groups despite not reaching statistical significance **(Figure 13A-B)**. This finding is suggestive of a potential role of ER-β attenuating HIF-1α accumulation in hypoxia. Similar pattern was also seen with HIF-3α **(Figure 14A-B).** This is of interest since some isoforms of HIF-3α are known HIF-1α inhibitors.

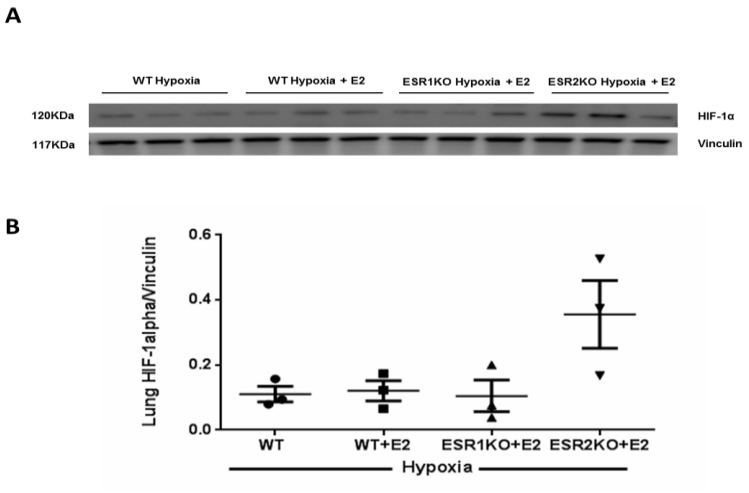

Figure 13. ER-β knockout (KO) results in accumulation of HIF-1α expression in the hypoxic lung A. HIF-1α protein expression in mouse lung homogenates from WT, WT+E2, ESR1 KO+E2, ESR2 KO+E2 mice exposed to 3 weeks of hypoxia. **B.** Expression levels of lung HIF-1α quantified by densitometry.

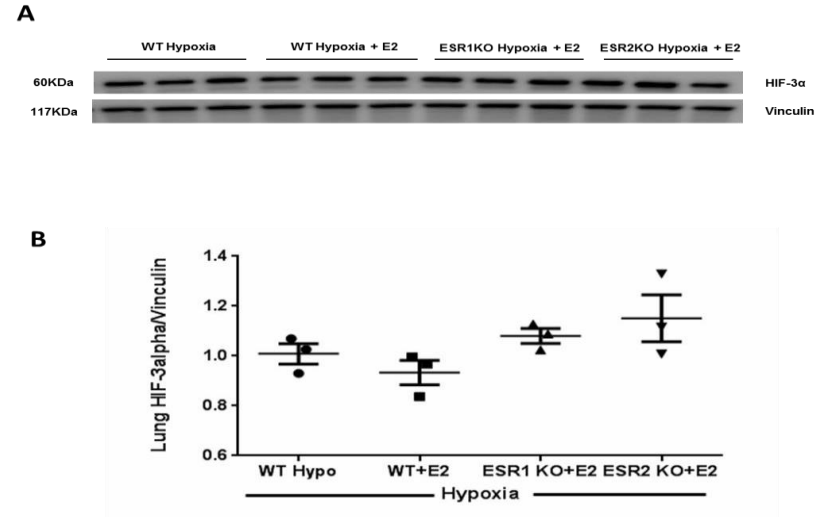

Figure 14. ER-β knockout (KO) results in accumulation of HIF-3α expression in the hypoxic lung. A. HIF-3α protein expression in mouse lung homogenates from WT, WT+E2, ESR1 KO+E2, ESR2 KO+E2 mice exposed to 3 weeks of hypoxia. **B.** Expression levels of lung HIF-3α quantified by densitometry.

IV. DISCUSSION

In hypoxic and monocrotaline PH animal models, work from our lab and others has shown that E2 attenuates as well as rescues PH endpoints; hemodynamics, proliferation and remodeling [37, 38]. This effect is believed to be endothelium-dependent and receptor-mediated [37]. Moreover, this protection is thought to be mediated mainly by ER-β as ER-β blockade attenuates E2's effects during hypoxia *in vivo* and in RPAECs [37]. Furthermore, selective estrogen receptor ER-β agonist diarylpropionitrile (DPN) is as effective as estrogen (E2) in rescuing severe monocrotaline induced- PH; an effect that is lost in the presence of an ER-β antagonist, PHTPP [38]. In isolated rat pulmonary artery rings, selective activation of ER-β attenuates hypoxic vasocontraction in an endothelium-dependent manner [31].

Various mechanisms have been proposed to explain E2 mediated protection in PH. These effects include suppression of inflammation [38, 76, 77], proliferation [37] and fibrosis [78, 79]. E2 is also thought to enhance lung autophagy [37], promote neoangiogenesis [38] as well as directly induce vasorelaxation of the pulmonary vasculature [29, 30]. On the molecular level, these effects are thought to be mediated *via* attenuation of ERK1/2 activation, increasing LC3-II expression and VEGF inhibition [37]. Interestingly, E2 mediates its protective effects from cardiac fibrosis *via* ER-β [78]. Pedram and colleagues showed that both E2 and ER-β agonist DPN mediated blockage and reversal of transition of cardiac fibroblasts to myofibroblasts and consequent fibrosis mediated *via* cAMP and protein kinase A resulting in inhibition of JNK activation. These effects were shown to be lost with ER-β knockdown as well as in an ER-β knockout model [78].

Cross talk between ER signaling, transcriptional activation and hypoxia-responsive pathways including HIF is not entirely understood. [70]. HIF-1 simulator, cobalt chloride ($CoCl_2$) was shown to increase ER-β expression in MCF7 human breast cancer cell line but not in MDA-MB231 cells [80]. Data in HEK293 cells revealed that HIF-1α transcriptionally activates ER-β *via* protein-protein interaction [71]. However, the unoccupied ER-β inhibits HIF-1 transcriptional activity *via* downregulation of ARNT (HIF-1β) [72]. This finding was replicated in human prostate cancer cell lines (PC3 cells) [81]; where knockdown of ER-β1 in PC3 cells resulted in HIF-1α accumulation and

epithelial-mesenchymal transition (EMT). Moreover, proteasome inhibition resulted in HIF-β accumulation in control but not in ER-β1 knockdown cells [82]. Work by the same group revealed that ER-β regulates HIF-1α *via* promoting prolyl hydroxylase-2 (PHD-2) transcription and consequently, HIF-1α degradation maintaining epithelial differentiation [83].

VEGF is a major modulator of hypoxia induced pulmonary vasculature remodeling [3]. Evidence exists to support that the non-ligand ER-β attenuates the hypoxic induction of HIF-1α mediated VEGF transcription [72, 81]. Lahm et al showed that E2 decreased VEGF secretion in hypoxic, but not normoxic rat PAECs in an ER-independent manner persisting despite ER blockade [37]. These reports further suggest that ligand-dependent ER activation that results in attenuation of HIF-1 downstream signaling is replicated in the pulmonary endothelium.

Our current data identifies ER-β as being distinctly up-regulated in the hypoxic lung and pulmonary vasculature. Despite no evidence to suggest hypoxia induced up-regulation of ER-β gene expression, other mechanisms of HIF-1α induced ER-β up-regulation could not be excluded. We hypothesized that this hypoxia-mediated ER-β up-regulation is HIF-1-dependent. Our data, however, indicates that HIF-1 in neither necessary nor sufficient to induced hypoxia-mediated ER-β up-regulation. Alternative mechanisms for ER-β over-expression during hypoxia include other transcription factors described to be involved in hypoxia like nuclear factor Kappa B (NF-kB), peroxisome proliferator-activated receptors (PPARs) and early growth response (Egr-1) [84-89]. Alternatively, other members of HIF family like HIF-2 and HIF-3 could be involved. As opposed to the ubiquitous HIF-1, HIF-2 is more endothelial cell specific. HIF-1α & HIF-2α activate HRE gene expression, have non-redundant roles and have different transcriptional activities [90-93]. Therefore, HIF-2α overexpression and its effect on ER-β expression are currently being investigated.

Functionally, our ER KO hypoxic model data highlight the significance of ER-β. These data reveals that in the absence of ER-β, E2 mediated attenuation of vascular remodeling and pro-proliferative signaling i.e. ERK1/2 activation, is lost. Moreover, these data identify the downregulation of HIF1-α as a mechanism of ER-β mediated protection in HPH. This result was demonstrated in our *in vivo* mouse model of hypoxic

PH; reflected by accumulation of HIF-1α only in ESR2 KO mice. Similar accumulation was seen in HIF-3α protein. It is known that hypoxia induces HIF-3 transcription and stabilization. HIF-3α is believed to be complementary, rather than redundant to HIF-1α, in protection against hypoxic damage in alveolar epithelial cells [94]. Certain isoforms of HIF-3α like HIF-3α2 and like HIF-3α4 [95] are known to have HIF-1 inhibitory effects.

This putative negative feedback of ER-β on HIF-1α is consistent with data from the oncology literature revealing that ER-β inhibits HIF-1 transcriptional activity as well as promotes its proteasome degradation in breast and prostate cancer cells [72, 81-83]. Exploring the cellular mechanism of ER-β attenuation of HIF-1, in the lung and RAPECS, is currently being investigated. In the context of HPH, achieving mechanistic understanding of hypoxia induced ER-β up-regulation as well ER-β regulation of the major hypoxia regulator HIF-1 aims to identify potential targets for non-hormonal PH therapeutics. A schematic of the putative interaction between hypoxia and its molecular responsive pathways, HIF-1 and ER-β is illustrated in **Figure 15.**

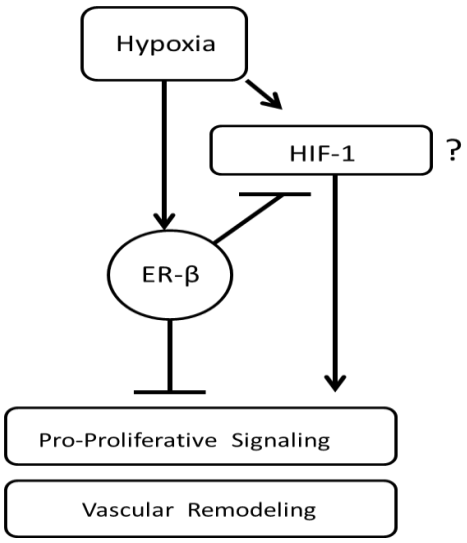

Figure 15. Schematic representation of the putative interaction between hypoxia, HIF-1 and ER-β.

V. FUTURE STUDIES

We plan to perform HIF-1α knockout in hypoxic RPAECs to evaluate the consequences of the loss of HIF-1 on hypoxia- induced ER-β up-regulation. Additionally, HIF-2α overexpression is to be utilized to study the effect of selective normoxic HIF-2α overexpression on ER-β expression in RPAECs. Selective ER-β overexpression and ER-β agonism with diarylpropionitrile (DPN) are planned to be utilized to study the effect of non- ligand ER-β overexpression and ligand-bound ER-β activation on HIF-1α protein level, HIF-1α transcriptional activation as well as ERK1/2 activation and VEGF levels in normoxic and hypoxic RPAECs. Other future directions include investigating PHD2 as a potential mechanism of ER-β induced HIF-1α degradation in the presence of DPN, ER-β overexpressing plasmid as well as in ESR2 KO mouse model. Moreover, studying the effect of hypoxia on ER-β nuclear translocation as well as the selective function of membrane and nuclear ER-β are exciting future frontiers to explore.

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CURRICULUM VITAE

MONA M.A. SELEJ

Education

Indiana University, Master of Science M.S., 07/2013
University of Jordan, Jordan-School of Medicine M.B.B.S., 06/2005 (GPA 3.94)

Certification/Licensure

California Medical License 5/2013
Indiana Medical License 7/2010
Internal Medicine Board Certification 8/2010
Pulmonary Medicine Board Certification 11/2012
ACLS, BLS 7/2006

Examinations

ABIM Internal Medicine Certification Examination 8/2010
ABIM Pulmonary Medicine Certification Exam 11/2012
USMLE Step 3 5/2009 (99th percentile)
USMLE Step 2 CS (Clinical Skills) 6/2006 (99th percentile)
USMLE Step 2 CK (Clinical Knowledge) 10/2005
USMLE Step 1 3/2006 (99th percentile)

Research Experience

7/2011-6/2013 **Postdoctoral fellow**
Indiana University; Division of Pulmonary, Allergy, Critical Care, Occupational and Sleep Medicine.
Mentors: **Tim Lahm, MD and Irina Petrache, MD.**
Project title: *"Effects of Hypoxia on Estrogen Receptor Expression in Rat and Human Pulmonary Artery Endothelial Cells".*

Postgraduate Training

7/2013-7/2014 **Pulmonary Vascular Diseases Fellowship**
Stanford University
Program Director: **Roham Zamanian, MD**

7/2010-6/2013 **Pulmonary Critical Care Fellowship**
Indiana University; Division of Pulmonary, Allergy, Critical Care, Occupational and Sleep Medicine; Indianapolis, IN
Program Director: **John Buckley, MD**

7/2008-6/2010	**Internal Medicine Residency** University of Arizona, Tucson, Arizona Program Director: **William Johnson, MD**
7/2007-6/2008	**Internal Medicine Internship** University of Arizona, Tucson, Arizona Program Director: **William Johnson, MD**
7/2006-6/2007	**Transitional Internal Medicine Residency** King Hussein Cancer Center, Amman, Jordan
7/2005-6/2006	**Internship** Jordan University Hospital and Ministry of Health Hospitals-Amman, Jordan

Published Research Manuscripts

1. T.Lahm, M.Albrecht, A.Fisher, **M. Selej**, N. Patel, J. Brown, M. Justice, M. Brown, M.Van Demark, K. Trulock, D. Dieudonne, Dino; J.Reddy, R. Presson, I. Petrache. "*17-Beta Estradiol Attenuates Hypoxic Pulmonary Hypertension Via Estrogen Receptor-Mediated Effects"* American Journal of Respiratory and Critical Care Medicine, 2012. 185(9): p. 965-80.

2. **M.Selej**, T.Lahm. *"Red card for white blood cells: leukocytapheresis in sepsis"* J Surg Res. 2011 Jul; 169(1):21-4.

Manuscripts in Preparation

Manuscript titled: "*Hypoxia Increases Lung ER-beta expression In Vivo and In Vitro*". It will include the results of my project studying the effects of hypoxia on rat pulmonary artery endothelial cells as well and lung and right ventricular tissue in a hypoxic pulmonary hypertension model.

Book Chapters

M.Selej and T.Lahm. "*Epidemiology and Genetics of Pulmonary Arterial Hypertension".* In: Sood N: Clinical Focus on Pulmonary Hypertension (in press).

Research Posters

1. **M. Selej**, J. Wood, A.D. Lockett, M. Albrecht, K. Schweitzer, I. Petrache, T. Lahm. "*Hypoxia Increases Expression of Estrogen Receptor (ER)-Beta in Vivo and In Vitro"* (Poster Discussion). American Thoracic Society International Conference, Philadelphia, PA, USA 2013.

2. **M. Selej**, A. Lockett, M. Albrecht, I. Petrache, T.Lahm. "*Hypoxia Increases Estrogen Receptor Beta Expression in Cultured Pulmonary Artery Cells"* (Poster). Pulmonary Hypertension Association (PHA) conference, Orlando, FL, USA 2012.

3. Lahm T, Albrecht M, Fisher A, **Selej M**, Patel N, Brown J, Justice M, Brown M, Trulock K, Dieudonne D, Reddy J, Presson R, Petrache I. "*Protective Effects of*

17-Beta Estradiol (E2) in Hypoxic Pulmonary Hypertension are Mediated by Estrogen Receptors Alpha and Beta" (Poster). Pulmonary Hypertension Association Scientific Sessions, Orlando, FL, USA 2012.

4. Lahm T, Albrecht M, Fisher A, **Selej M**, Patel N, Brown J, Justice M, Brown M, Trulock K, Dieudonne D, Reddy J, Presson R, Petrache I. *"Protective Effects of 17-Beta Estradiol (E2) in Hypoxic Pulmonary Hypertension are Mediated by Estrogen Receptors Alpha and Beta"* (Poster). Aspen Lung Conference, Aspen, CO, USA 2012. (Abstract in press in *Pulmonary Circulation*).

5. **M.Selej**, A. Lockett, I. Petrache, T.Lahm. "*Hypoxia Increases Estrogen Receptor Beta Expression in Cultured Pulmonary Artery Cells"* (Poster). American Thoracic Society International Conference, San Francisco, CA, USA 2012.

6. T. Lahm, M. Albrecht, A.Fisher, N. Patel, M. Justice, **M.Selej**, R.Presson, I. Petrache. "*17β Estradiol (E2) Attenuates Hypoxia-Induced Pulmonary Hypertension (HPH) Through an Estrogen Receptor-Dependent Mechanism That Involves Decreased ERK1/2 Activation*" (Poster). Grover Conference on Pulmonary Vascular Disease, Sedalia, CO, USA 2011 (Abstract in press in *Pulmonary Circulation*).

Clinical Oral Presentations and Posters

Oral Presentations

1. **M.Selej**, B.A. Khan, S. Zielinski, C. Naum. **"***Secondary Pulmonary Alveolar Proteinosis and Hay Dust Exposure in a Farmer*" at American Thoracic Society Conference, Denver, CO, USA 2011.

Clinical Posters

1. A. Bitar, **M.Selej**, V.Williams, I.Bolad, T.lahm. *"Poor* Agreement between Pulmonary Capillary Wedge Pressure (PCWP) and Left Ventricular End-Diastolic Pressure (LVEDP) In A Veteran Population" (Poster). American Thoracic Society International Conference, Philadelphia, PA, USA 2013.

2. J.Smith, **M.Selej**, W. Carlos, K.Diab*. "A Case of Mediastinitis Following Endobronchial Ultrasound Guided Transbronchial Needle Aspiration"* (Poster). American Thoracic Society International Conference, Philadelphia, PA, USA 2013.

3. **M.Selej**. "*ALCAPA: A Rare Cause of Cardiac Arrest"* (Poster). American College of Chest Physicians- ACCP conference, Atlanta, Georgia, USA 2012.

4. K.Van Nostrand, **M.Selej,** B.Khan, A.Noor. *"Lymphocytic Alveolitis Complicating Erythodermic Psoriasis"* (Poster). American Thoracic Society International Conference, San Francisco, CA, USA 2012.

5. **M.Selej**, M.Farber. "*Noncardiogenic pulmonary edema in Amlodipine overdose*" (Poster). American College of Chest Physicians- ACCP Conference, Honolulu, HI, USA 2011.
6. **M.Selej**, G.Bosslet. *"Sarcoidosis: A Great Imitator*" (Poster). American College of Chest Physicians- ACCP Conference, Honolulu, HI, USA 2011.

7. **M. Selej**, B.A. Khan, S. Zielinski, C. Naum**. "***Secondary Pulmonary Alveolar Proteinosis and Hay Dust Exposure in a Farmer*" (Poster) American Thoracic Society International Conference, Denver, CO, USA 2011.

8. **M.Selej**. "*Myxedema Coma: Not Merely a Textbook Myth"* (Poster). American College of Physicians, Arizona Chapter- clinical vignettes' session. Phoenix, AZ, USA 2009.

Awards

1. Indiana University Annual Fellows' Research Competition - 2013 (Second Place).
2. American College of Chest Physicians (ACCP) Fellows Asthma Conference Awardee, Atlanta, GA, USA 2012.
3. Pulmonary Hypertension Association (PHA) Fellows Travel Awardee, Orlando, FL, USA 2012.

Conferences Attended

ATS- Philadelphia, PA 2013
CHEST-Atlanta, GA 2012
PHA -Orlando, FL 2012
ATS-San Francisco, CA 2012
CHEST- Honolulu, HI 2011
Grover Conference – Sedalia, CO 2011
ATS - Denver, CO 2011

Membership in Honorary / Professional Societies

American Medical Association (AMA) 2007
American Thoracic Society (ATS) 2010
American College of Chest Physicians (ACCP) 2010
Society of Critical Care Medicine (SCCM) 2010
Pulmonary Hypertension Association (PHA) 2012