Effect of Estrogen Receptor Activation on Expression of Genes Indicated in Pulmonary Fibrosis

Lauren F. Robertson

University of Florida

Abstract

Idiopathic pulmonary fibrosis (IPF) is a chronic disease in which normal lung tissue is replaced with connective tissue from scarring partly due to lung inflammation. Men are disproportionally affected, with evidence suggesting that estrogen may play a protective role in IPF progression through activation of estrogen receptors (ERα and ERβ). However, the connection between gender, IPF development, and its mechanism is unclear. In this study, we performed a 24-hour exposure of human bronchial epithelial cells to one of three treatments: the normal endogenous estrogen 17-β estradiol (E2), ERα agonist propylpyrazole-triol (PPT), or ERβ agonist diarylpropionitrile (DPN). We expected the treatment groups to have altered expression of genes indicated in IPF compared to controls. After collecting mRNA from the exposed cells, qPCR was performed to quantify gene expression differences between control and treatment cells. Results show significant differences for two genes: fibronectin 1 (*FN1*) and retinol binding protein 7 (*RBP7*). Both DPN and PPT increased expression of *FN1*, and all treatment groups showed increased expression of *RBP7*. These genes code for proteins which play a role in the onset and progression of fibrotic disease, suggesting that estrogen may have a direct influence on its development.

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Indicated in Pulmonary Fibrosis

It is apparent that there is a difference between male and female incidence and prevalence of pulmonary fibrosis, both in mice/rat models and in humans. However, it is unclear how sex hormones may play a role in pulmonary fibrosis development and progression. Studying the results of estrogen receptor activation may provide initial clues regarding the role that estrogen may play in fibrotic disease.

Introduction

Pulmonary fibrosis is a progressive, generally fatal disease characterized by scarring of lung tissue. When there is not a known cause, which is commonly the case, it is called idiopathic pulmonary fibrosis (IPF). This scarring results in decreased function of the lungs and reduced oxygen in the bloodstream. Characteristics of pulmonary fibrosis include altered lung fibroblasts, loss of alveolar epithelial cells, and excessive accumulation of extracellular matrix (Todd, Luzina, & Atamas, 2012). Of over 150 identified types of interstitial lung disease, IPF is the most prevalent with the highest mortality rate (Raghu, Weycker, Edelsberg, Bradford, & Oster, 2006): after diagnosis, the reported median survival time is three to six years (Bjoraker et al., 1997). Between 1992 and 2003, the age-adjusted mortality rate of patients with pulmonary fibrosis increased 28.4% in men and 41.3% in women (Olson et al., 2007). Although women saw a steeper increase in mortality rate, men still have higher incidence and mortality rates of IPF (Cary et al., 2007). Using data from ten different countries, it was found that mortality from pulmonary fibrosis is increasing worldwide, expecting to double in 36 years (Hutchinson, McKeever, Fogarty, Navaratnam, & Hubbard, 2014). This data shows an increasing incidence and prevalence of IPF worldwide, as well as the severity of this condition.

Currently, the role that sex hormones and gender play in fibrosis development and the response to lung injury is unclear. However, estrogens are known to affect alveolar and lung development (Carey et al., 2007), and there are estrogen and androgen receptors in respiratory

neurons (Behan & Thomas, 2005). A study by Haston et al. (2002) found that male and female rats exhibited different susceptibility to bleomycin-induced lung fibrosis, suggesting sex-specific models. Other studies in rats show that females have both higher mortality rates and more severe fibrosis than males (Gharaee-Kermani, Hatano, Nozaki, & Phan, 2005). However, there have also been studies suggesting that estrogen may play a protective role in IPF (Lekgabe et al., 2006) and studies suggesting that male sex hormones may exacerbate lung function impairment in IPF (Voltz et al., 2008). Although it is clear that sex hormones play a role in the development of IPF, there is still uncertainty as to how.

With further research on the role of sex hormones in IPF, Gharaee-Kermani et al. (2005) found that estradiol replacement in ovariectomized female rats restored fibrotic lung response that had been diminished in ovariectomized rats without hormone replacement. In addition, fibroblasts from the rats without hormone replacement showed an increased responsiveness in pathways leading to fibrosis when treated with estradiol.

Estrogen binds and activates a series of nuclear receptor termed ERα and ERβ. Past research has shown that these receptors can work both together and independently to control the expression of downstream genes. Few studies have examined the role of estrogen and these receptors in the lung and specifically in the context of IPF. One study performed by Carey et al. (2007) found that ERβ-deficient mice had alveolar abnormalities, highlighting the importance of this receptor in lung development. The ability to activate each receptor separately allows for the study of the interaction between the two types of receptors. Song and Pan (2012) used an ERα-selective agonist and an ERβ-selective agonist to find evidence supporting that ERα activity can be opposed by the ERβ-selective agonist. The ERα-selective agonist used was propylpyrazole-triol (PPT), and the ERβ-selective agonist used was diarylpropionitrile (DPN).

There are multiple genes suspected to be involved in IPF and fibrotic activity. Two genes of interest in this study are fibronectin 1 (*FN1*) and retinol binding protein 7 (*RBP7*). *FN1* codes for fibronectin, which is a protein produced in response to early inflammation in lung injury

(Hernnas et al., 1992). Fibronectin contributes to the creation of fibers and extracellular matrix, and increased levels of fibronectin is an early sign of lung inflammation, leading to the development of IPF. In patients with IPF, there is a significant elevation of fibronectin release by alveolar macrophages compared to patients without IPF (Lacronique, Rennard, Bitterman, Ozaki, & Crystal, 1984).

RBP7 encodes for a protein in the cellular retinol-binding protein (RBP) family, indicated in extracellular transport of retinol (NCBI, 2018). Emblom-Callahan et al. (2010) found that alterations in retinol transport affects the citric acid cycle, influencing cellular energetics. This may influence growth of IPF fibroblasts, and this study found that *RBP7* was down-regulated in IPF fibroblasts. However, in a study by Molyneaux et al. (2017), it was shown that *RBP7* is up-regulated in IPF lung tissue. This was also shown in wound tissue in the wound model of the normal chicken chorioallantoic membrane (Soulet et al., 2010). In addition, increased levels of urinary RBP is positively correlated with interstitial fibrosis (Pallet et al., 2014). Retinoic acid is a metabolite of retinol, and is involved in various developmental and growth processes. However, there is conflicting research on the effect that retinoic acid has on collagen production and fibrosis, possibly due to studies using different isoforms and doses of retinoic acid (Zhou, Drummen, & Qin, 2013). Thus the role of *RBP7* in fibrosis is still unclear.

Other genes possibly involved in pro-fibrotic development include *VIM* and *MMP7*. Vimentin, the protein produced by *VIM*, has been shown to be a key regulator in inflammation and fibrotic development (dos Santos et al., 2015). *MMP7* has been shown to be consistently up-regulated in IPF lungs, and *MMP7* null mice are protected from bleomycin-induced lung fibrosis (Pardo & Selman, 2012). These are just a few examples of genes that have been shown to increase fibrotic signaling, and may be involved in the development and severity of IPF.

Studying the action and targets of estrogen in the lungs will provide better understanding on how estrogen can affect IPF development and advance our understanding of disease

mechanisms. This may lead to better, more effective diagnostic and individualized and targeted therapeutic strategies for future patients.

Aims and Hypotheses

- The aim of my study was to determine the transcriptional regulation of pulmonary fibrosis-related genes by the specific ER subtypes.
- It is anticipated that ERα and ERβ activation may differentially modulate gene expression, providing initial evidence for estrogen involvement in the disease.
- We aim to identify targets of estrogen in the lungs, specifically in pro-fibrotic genes indicated in pulmonary fibrosis.

Methods

The independent measure in this study was estrogen receptor isotype activation and the resultant downstream gene expression. We utilized commercially available agonists to activate each ER; PPT has ERα-selective activation, DPN has ERβ-selective activation, and the 17β estradiol (E2) activates both ERα and ERβ. The dependent measure was the resulting fold change expression of the tested genes. There were eleven gene targets tested in total, listed in table 1.

Table 1

List of Genes Tested and Their Indication in Idiopathic Pulmonary Fibrosis (IPF), (NCBI, 2018)

Gene	Name	Function
RBP7	Retinol binding protein 7	Codes for retinol-binding protein, required for vitamin A stability and metabolism
FN1	Fibronectin 1	Codes for fibronectin, a protein involved in cell adhesion, wound healing, and metastasis
CDH1	Cadherin 1	Codes for cadherin, a protein involved in cell-to-cell adhesion, represses proliferation/metastasis

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Gene	Name	Function
SNAI1	Snail family transcriptional repressor 1	Down-regulates expression of ectodermal genes within the mesoderm
MMPs 2 and 7	Matrix metallopeptidases 2 and 7	Cleaves components of the extracellular matrix and signal transduction molecules, involved in metastasis
VIM	Vimentin	Encodes a type III intermediate filament protein, involved in cell attachment and signaling
SMADs 2, 3, 4, and 7	SMAD family members 2, 3, 4, and 7	Code for SMAD proteins, which are signal transducers and transcriptional modulators, regulating proliferation and differentiation

Note. Data in this table is collected from the National Center for Biotechnology Information's Gene Database (2018).

This experiment was performed using immortalized human bronchial epithelial cells, known as BEAS-2B cells. The 12-well plate used was coated with 450 µL of collagen matrix for 24 hours, then a total of 400,000 cells was placed in each well. Cells were then exposed to 2 mL of either 10 nM estradiol (E2), 100 nM PPT, or 100 nM DPN for 24 hours. The control wells were exposed to 1 µL DMSO to 1 mL of media. Before the treatment, the cells used were observed to be relatively confluent. After the 24 hours, media was removed from the wells and 750 µL of STAT-60 was added to each well, pipetting along sides and bottoms of the wells to detach the cells. Then the STAT-60 and cell mixture was added into a 2 mL tube for each sample, and mRNA was collected from the cells using lab-established protocol.

mRNA Isolation

To extract the mRNA from each sample, samples were vortexed and sat at room temperature for 5 minutes to allow for complete dissociation of nucleoprotein complexes. 150 µL of molecular-grade chloroform was added to each sample. After sitting for another 2 to 3 minutes at room temperature, samples were put in the centrifuge for 15 minutes at 14,000 rpm at 4°C. This separates the homogenate into three separate phases. The aqueous phase,

containing the RNA, was transferred to a clean tube containing 750 µL STAT-60. 150 µL of chloroform was added, and spinning in the centrifuge was repeated. The aqueous phase from this second extraction was transferred into clean tubes, then 1 µL of glycol blue was added. This was used to help increase yield. After adding 500 µL of molecular-grade isopropanol to each sample, samples were placed in a -20°C freezer overnight.

The next day, samples were placed in the centrifuge for 30 minutes at 14,000 rpm at 4°C, and RNA formed blue pellets. However, pellets were not observed in samples 1, 2, 3, and 12. This was all three control samples, as well as one of the E2 samples. Supernatant was removed, and the pellets were washed with 750 µL of 70% molecular-grade ethanol. The samples were then placed in the centrifuge again, but for 5 minutes. The ethanol wash/centrifuge step was repeated, then all ethanol was removed. The samples air dried until the pellets were clear. While the samples were drying, Ambion RNAsecure was pre-heated in a 60°C heating block. 15 µL of RNAsecure was added to each sample - just enough for the pellet to dissolve. After heating for 2 minutes, samples were vortexed and returned to the heating block to incubate for 10 minutes to inactivate RNase contamination. The BioTek plate reader was used to determine nucleic acid concentration and RNA quality in each sample. At this point in the extraction process, it was determined that mRNA collected from sample 9, which was in the PPT treatment group, was unusable, leaving the sample size for that treatment group at two, rather than three.

For the DNase I treatment, the final sample reaction volume used was 10 µL. This was from a combination of 1 µL of 10X reaction buffer, 1 µL of PerfeCta DNase I (2U/µL), RNase/ DNase-free water, and the RNA template. Then, each sample was gently vortexed and placed in the centrifuge to collect contents at the bottom of the tube. Samples were incubated for 30 minutes at 37°C, then 1 µL of 10X stop buffer was added, and samples were incubated for 10 minutes at 65°C.

cDNA Synthesis

To synthesize cDNA, 1 µg of RNA was combined with nuclease-free water to form 15 µL. Then, 4 µL of 5X qScript reaction mix was added, along with 1 µL of qScript RT. After each sample was vortexed, they were placed in the thermal cycler programmed as follows: one cycle at 22°C for 5 minutes, one cycle at 42°C for 30 minutes, one cycle at 85°C for 5 minutes, then hold at 4°C. This completed the cDNA synthesis.

Measuring Gene Expression

With the collected cDNA diluted to 1:20, qPCR was run to test eleven different genes indicated by previous studies to affect pulmonary fibrosis. Expression of GAPDH was used as a housekeeping gene. From the collected data, the fold change in expression compared to the control was determined using the delta Cq method and used to analyze gene expression changes.

Statistical Analysis

To determine the fold change for each gene, data was entered into a spreadsheet in Microsoft Excel. The Cq for each sample (the cycle in which fluorescence can be detected) was recorded, and normalized with the Cq for the housekeeping gene, GAPDH. This was done by taking the difference between the sample's Cq for the gene in consideration and the sample's Cq for GAPDH. This gives the ΔCq for each sample. Then, the difference of the ΔCq for each sample from the highest ΔCq of the group was done for each group. Log_2 of this number gives the fold change for each sample.

To analyze the results and determine statistical significance, the computer program Prism 6 was used. Each of the eleven genes studied were organized individually in separate data tables. The fold change for each sample was entered for each gene into the data tables, organized by treatment group and unpaired t-tests were run. This compared each treatment group's fold change data to the control individually. Because each group had a sample size of three or less, there is no normality.

Results

Estrogen receptor activation was shown to have a statistically significant effect on gene expression of two of the eleven genes: *FN1* and *RBP7*. For *FN1*, there was a statistically significant increase in fold change between the control and DPN ($p<0.01$), as well as the control and PPT ($p<0.02$) (see figure 1). For *RPB7*, there was a statistically significant increase in fold change between the control and each of the treatment groups (DPN $p<0.05$, PPT $p<0.03$, E2 $p<0.01$) (see figure 2).

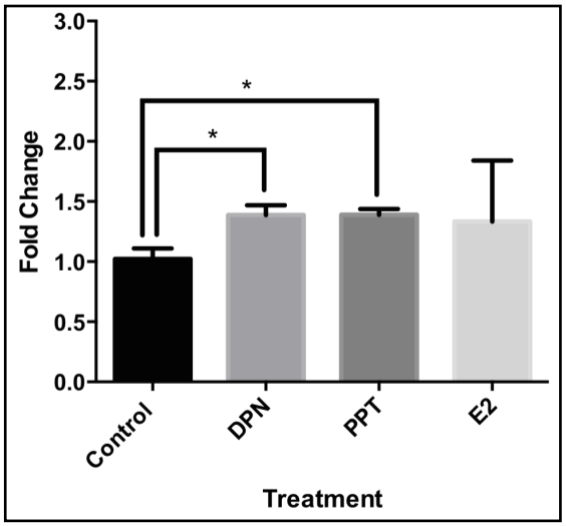

Figure 1. Fold change in *FN1* expression based on estrogen receptor activation. P<0.02 (*)

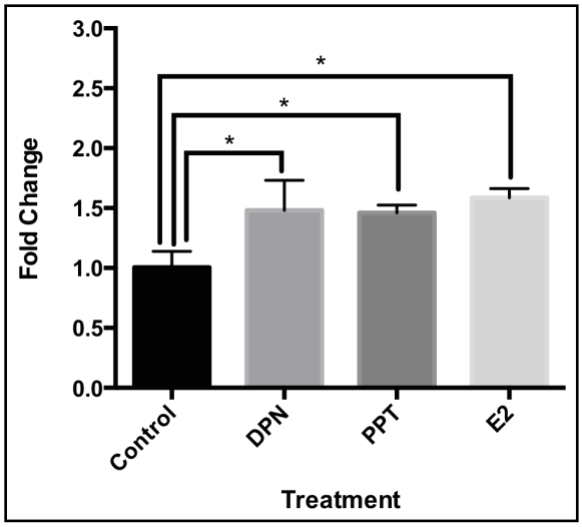

Figure 2. Fold change in *RBP7* expression based on estrogen receptor activation. P<0.05

When considering the results for *FN1* expression, there is no statistically significant difference in gene expression between the E2 treatment group and the control group. This shows that activation of the ERs may affect gene expression differently: ERα and ERβ simultaneous activation had no statistically significant impact on gene expression, whereas ERα and ERβ activation individually did. However, in *RBP7* expression, this was not exactly the case. The E2 group showed a significant increase in expression when compared to the control, as well as the DPN and PPT groups.

When designing this experiment, there were eleven genes selected due to their potential involvement in fibrosis development and IPF markers. Out of these eleven genes, this study

showed that *FN1* and *RBP7* appeared to be the only ones affected by ER activation. The fold change differences between the treatment groups for the two genes are similar as well. For both DPN and PPT groups in each gene, fold change was increased to roughly 1.5. Although these are the two genes that saw a statistically significant fold change, there are two other genes that show a possible trend. However, small sample sizes resulted in larger measures of error. If there were more repetitions of this study, there may be statistical significance in changes in expression of these genes.

VIM expression showed an increased trend in expression for the treatment groups (see figure 3). This increase in expression was most clear for the PPT and E2 groups.

MMP7 is another gene which showed a general trend with reduced expression by the ER agonists (see figure 4). With further repetitions and more data, the trend in samples may be statistically significant.

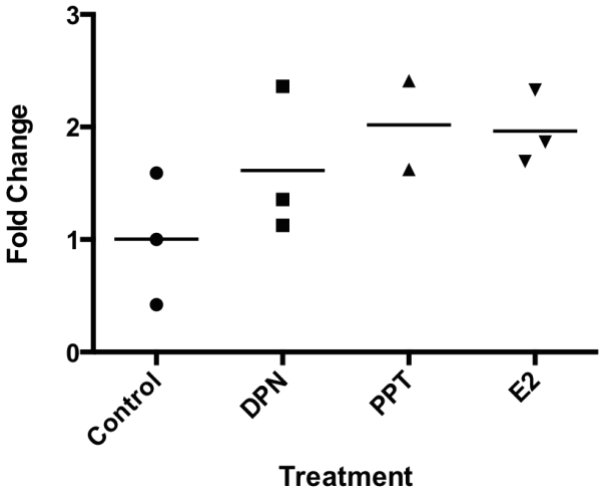

Figure 3. Fold change in *VIM* expression based on estrogen receptor activation. Points on the graph represent fold change values for each sample.

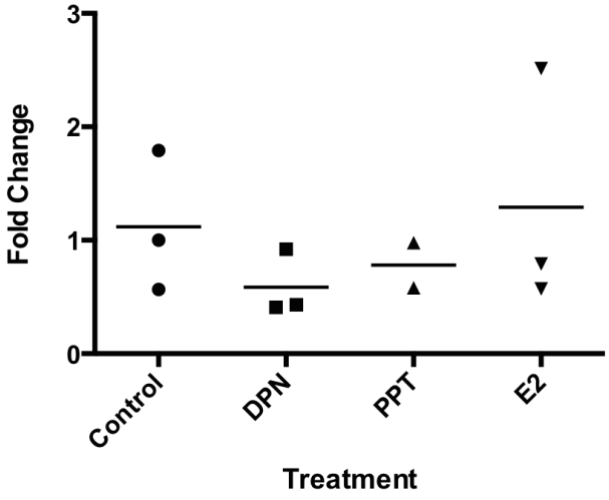

Figure 4. Fold change in *MMP7* expression based on estrogen receptor activation. Points on the graph represent the fold change values

Discussion

Data from this exposure suggest that activation of estrogen receptors by endogenous estrogen (E2) or select chemical agonists can alter the expression of genes known to be involved in IPF and other lung diseases.

The results from the fold change of *FN1* expression imply that ERα-selective and ERβ-selective activation increase gene expression. However, ERα and ERβ may counteract each other when activated simultaneously, because there was no significant difference between the E2 group and the control group. This supports findings by Song and Pan (2012) that the ERβ-selective agonist DPN can oppose effects from ERα-selective agonist PPT. This trend is also suggested in *MMP7* data. It is possible that ERα-selective activation would decrease expression of *MMP7*, but activating ERβ as well opposes this.

Overall, these results support findings from Gharaee-Kermani et al. (2005), Molyneaux et al. (2017), and Soulet et al. (2010). Findings that estradiol replacement restored a fibrotic tissue response in the lungs is supported by this study, as estrogen would activate the ERs, which modulates expression of pro-fibrotic genes. However, this contradicts current statistics that men experience higher rates of incidence and prevalence of IPF, as well as research by Lekgabe et al. (2006) suggesting that estrogen plays a protective role in IPF.

Given the statistically significant increase in fold change of *FN1*, and possible increase in *VIM*, this study supports ER activation leading to a pro-fibrogenic response. The up-regulation of *FN1* in IPF lungs has been largely supported, and *RBP7* has also been suggested as potentially involved in development of IPF in studies by Molyneaux et al. (2007) and Emblom-Callahan et al. (2010). Showing that estrogen can modulate *FN1* and *RBP7* expression also supports these genes as targets for estrogen in the lungs.

Study Limitations

However, there are limitations to this study. The media used to grow the BEAS-2B cells was backordered for much of the duration of the study, so that presented limitations to exposure repetitions. Because the study was only performed once, with two to three samples per

treatment group, it is possible that these results are not replicable. In addition, the small sample size of each treatment group, and thus low statistical power, presents limitations to the significance of the data.

While preparing the cells for exposure, cells were passaged multiple times due to insufficient growth. This may have led to abnormalities in the cell line used. These are all important considerations when presenting this data. In addition, although data show a statistically significant increase in gene expression, the fold change compared to the control is roughly 1.5. This may be a statistically significant difference, but whether or not it actually results in a physical difference and is biologically significant in lung cells is unknown.

Future Research

This is the first study to connect IPF-related genes and estrogens. This opens the door to future studies on the contribution that sex hormones may have on the development and severity of IPF. The next steps to consider would be to perform multiple repetitions of this exposure with more samples. Having more data to compare this to will give a clearer, more accurate depiction of whether or not ER-activation truly modulates the expression of genes indicated in IPF. In addition, previous research, such as that by Voltz et al. (2008), has presented the possibility of androgen involvement in IPF development. Possible exposures considering the difference between estrogen and androgen exposure to BEAS-2Bs would provide better data when comparing gender differences in IPF.

Moving forward, it is important to look more deeply into the impact that sex hormones have on IPF development. A clear difference in the population between genders raises the question of why and how these trends exist. A better understanding of this will allow us to develop more effective treatments and diagnostics, targeting the effects caused by sex hormones.

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