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Regulation of Endothelial Nitric Oxide Synthase in Pulmonary Myofibroblasts

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REGULATION OF ENDOTHELIAL NITRIC OXIDE SYNTHASE LOCALIZATION IN PULMONARY MYOFIBROBLASTS

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REGULATION OF ENDOTHELIAL NITRIC OXIDE SYNTHASE LOCALIZATION IN PULMONARY MYOFIBROBLASTS

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Idiopathic pulmonary fibrosis (IPF) is an interstitial lung disease leading to decreased lung volume and eventual respiratory failure. At present, the median post-diagnosis lifespan is between three and six years. Myofibroblasts are collagen-secreting cells essential for wound healing, but also implicated in the fibroproliferation and extra cellular matrix deposition commonly seen in IPF. The nitric oxide (NO) signaling pathway is implicated in protomyofibroblast to myofibroblast transition and regulation. Previous work has shown that in pulmonary myofibroblasts, endothelial nitric oxide synthase (eNOS) is the primary NOS isoform expressed. The current study used cultured rat pulmonary myofibroblasts between passages two and five as a cell model. The cells were grown in normal growth media (DMEM + 10% FBS) or serum starved (DMEM + 0% FBS) to induce cellular differentiation. In this study, immunocytochemistry was used to show localization of eNOS is dependent on cellular differentiation, with protomyofibroblasts expressing eNOS primarily in the nucleus and protomyofibroblasts expressing eNOS in the perinuclear region. We also show catalytic activity and localization of eNOS are correlated by visualizing nitric oxide production in the cells using a permeable fluorescein chromophore. By using western blot analysis on fractionated cell lysates we found eNOS expressed in

the nucleus under normal growth conditions. eNOS is at least partially regulated by intracellular calcium (Ca^{2+}) and calmodulin (CaM). Western blot analysis using native eNOS and phospho-specific eNOS antibodies on fractionated cells treated with the protein kinase C (PKC) activator phorbal 12-myristate 13-acetate (PMA) with and without addition of its antagonist ethylene glycol tetraacetic acid (EGTA) was conducted to investigate PKC's role in eNOS regulation by phosphorylation. Indeed, PKC activation was found to mitigate expression in the nucleus, while inhibition of the activator restored the activity expression above basal levels. This finding correlates with previous data from our lab showing a decrease in activity in myofibroblasts treated with PMA and assayed amperometrically with an NO electrode.

INTRODUCTION

Idiopathic Pulmonary Fibrosis

Idiopathic pulmonary fibrosis (IPF) is characterized by actively proliferating myofibroblasts and a subsequent uncontrolled release of extracellular matrix (ECM) proteins in pulmonary tissue, driving the destruction of the lung parenchyma architecture (Selman et al., 2001). Exertional dyspnea and chronic cough account for around 90% of the initial symptoms, and diagnostic confirmation is typically performed via a lung biopsy and chest radiographs, which show bilateral peripheral based reticular opacities and honeycombing predominately in the lower lobes (Figure 1). The American Thoracic Society and European Respiratory Society established international guidelines in 1999 regarding the diagnostic criteria that must be present for the confirmation of IPF. Briefly, a definitive diagnosis can be established in the presence of a surgical biopsy that includes the following:

1. Exclusions of other known causes of interstitial lung disease such as drug toxicities, environmental exposures, and collagen vascular diseases
2. Abnormal pulmonary function studies that include evidence of restriction and/or impaired gas exchange with rest or exercise or decreased carbon dioxide diffusing capacity of the lung
3. Abnormalities on conventional chest radiographs or high-resolution computed tomography scans (IPF: Diagnosis and Treatment 1999)

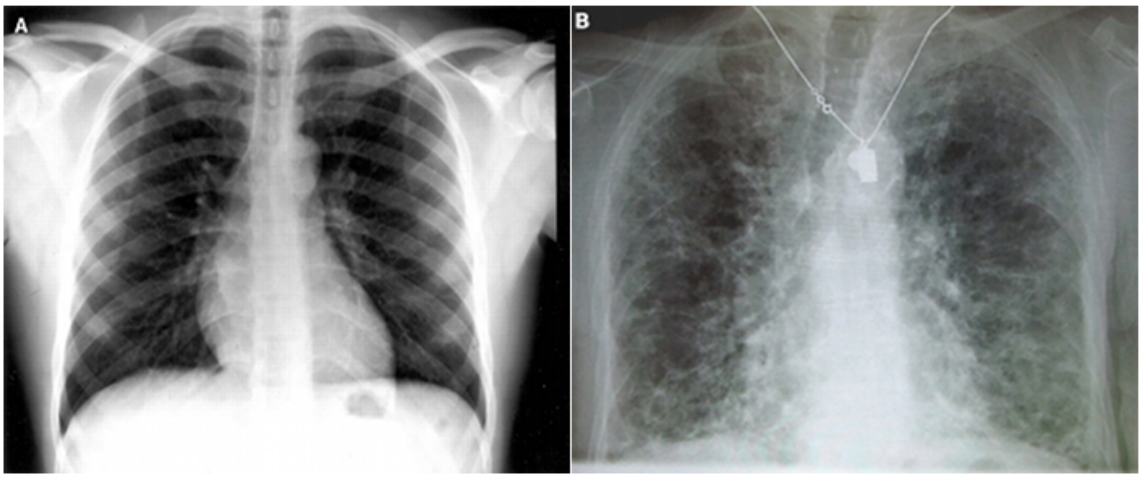

Figure 1: (A) Normal lung radiograph (BMJ publishing group, 2002). (B) Radiograph of patient with IPF. Bilateral scarring and honeycombing is prominent (Heilman, 2010)

Prevalence based on a cohort study from the years 1996 to 2000 was found to range from 4.0 per 100,000 persons aged 18-34 years to 227.2 per 100,000 persons among those 75 years and older (Raghu et al., 2006). Prognosis for those suffering from IPF, while displaying a fairly large heterogeneity depending on the time of diagnosis and subsequent treatment, is generally poor, with a post-diagnosis life span of three to six years (Bjoraker et al., 1998; Johnston et al., 1997; Gay et al., 1998).

IPF is thought to be a deleterious consequence of unresolved pulmonary inflammation that can be stimulated from various causative agents, including preexisting disease, acute illness and injury. Normally, patients are treated with anti-inflammatory therapies and show improvement in the days to weeks following diagnosis. However, a small number of these patients will progress towards chronic fibrosis, eventually showing the excessive ECM deposition and honeycombing of the lung. Patients presenting with IPF only respond mildly at

best to treatment with anti-inflammatory agents such as corticosteroids (Selman et al., 2002). It should be noted, however, that early *in vivo* characteristics of IPF show similarities to non-pathogenic usual interstitial pneumonia (UIP), so much so, in fact, that early stage identification of IPF cannot be readily distinguished from UIP (Nicholson et al., 2000). The unfortunate distinction that separates patients suffering from true IPF from those with UIP is death following the lack of physiological response to currently accepted treatment measures with high dose corticosteroids, such as prednisone and a supplemental cytotoxic immunosuppresent (Rhagu, 2006; Rudd et al., 1981; Johnston et al., 1991). This resistance to conventional treatment demands investigation into alternative therapeutic targets.

Cell biology of the myofibroblast

As a primitive mesenchyme-derived cell, myofibroblasts are a key part of normal tissue development. The initial identification of the myofibroblast came via electron microscopy, and was seen in the granulation tissue of healing wounds as a modulated fibroblast (Desmoulière & Gabbiani, 1996). This fibroblast-like cell exhibited features of smooth muscle cells, including bundles of microfilaments with dense bodies scattered in between and gap junctions (Desmouliere et al., 2007). Myofibroblastic modulation of fibroblastic cells begins with the appearance of the protomyofibroblast. Differentiation from protomyofibroblast to myofibroblast is characterized by the expression of alpha-smooth muscle actin (α-SMA) in the cell. The appearance of these

microfilaments with dense bodies suggests that myofibroblasts are responsible for the production of the force causing wound contraction (Desmouliere & Gabbiani, 1996). The mechanism behind the further modification of the protomyofibroblast to myofibroblast is not well known, but evidence has been shown correlating the differentiation with the production of transforming growth factor-beta (TGF-β) by inflammatory cells as well as products of other fibroblasts (See Figure 2) (Desmouliere et al., 1993). In the lung in particular, they play an important role in alveolar development and are necessary for the proper repair of tissues during wound healing and general tissue homeostasis (Walker, et al 2000). During wound healing, repair takes place in a well known, linear fashion: clots form, inflammatory cells invade injured tissues, fibroblasts migrate and begin the proliferation of extracellular matrix (ECM) components, the wound closes and, following the degradation of ECM, a scar forms (Desmoulière & Gabbinani, 1996).

While myofibroblasts are typically identified by α-SMA expression, expression of other sarcomeric proteins should be considered for their role in the myofibroblast's contractile funtions. Rice and Leinwand (2003) demonstrated that pulmonary myofibroblasts express three of the six skeletal isoforms of heavy chain myosin, including two adult isoforms, IIa and IId, and the developmental embryonic isoform. Inhibition of skeletal myosin activity significantly reduces contractile ability in the myofibroblast, an essential function in their ability to generate tensile forces in wound healing and contracture (Rice & Leinwand, 2003).

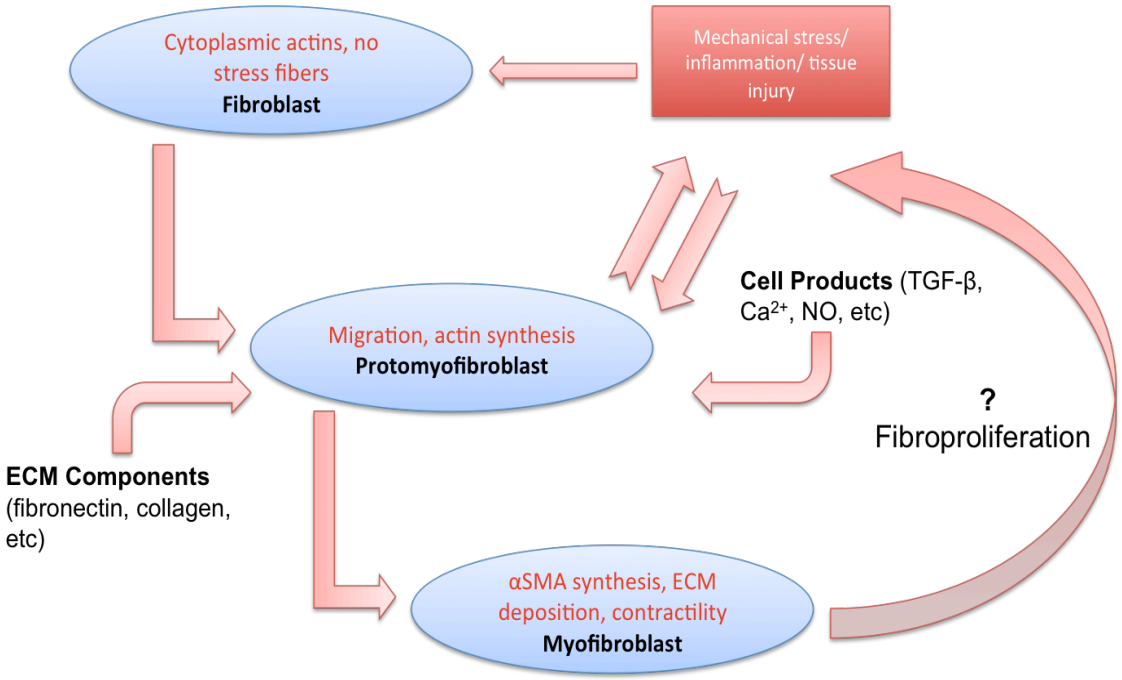

Figure 2: Fibroblast to myofibroblast transition **(**Adapted from Desmoulière et al., 2005**)**

The myofibroblast and IPF

In 1991, Kuhn and McDonald observed a matrix of fibronectin-containing fibrils, and among them myofibroblasts with well-formed actin filament bundles in biopsied lung tissue of seven patients suffering from usual interstitial pneumonia or bronchiolitis obliterans organizing pneumonia. These fibronectin-containing fibrils linked cells and collagen bundles and were structurally similar to the contractile phase seen in wound healing. This provided evidence that active fibroblast contraction plays a role in the remodeling lung tissue during pulmonary fibrosis (Kuhn & McDonald, 1991).

Further evidence of the fibroblast/myofibroblast role in IPF has been demonstrated by microarray analysis of pulmonary tissue from bleomycin-induced fibrotic mice. A drastic increase was noted in genes expressing

inflammatory mediators, components of the ECM and transforming growth factor-β1 (TGF-β1). The murine evidence was taken into consideration in the further identification of increased gene expression among tissue samples taken from five patients afflicted with IPF. In correlation with the IPF animal model, genes encoding proteins expressed in smooth muscle differentiation and muscle contractile machinery were identified in the samples, representing transcriptional signals of myofibroblasts and fibroblasts in the myofibroblast/fibroblast foci typically seen in the disease. Also observed was an increased transcription of genes encoding ECM proteins in fibrotic lungs (Kaminski, IPF supplement 2003).

Histologically, IPF is distinct from other idiopathic interstitial pneumonias in that it is a temporally heterogeneous disease. Temporal heterogeneity indicates various levels of injury within the same tissue biopsy, as opposed to temporal homogeneity, in which the age of lung injury is approximately the same in each tissue sample. Biopsies of IPF-associated lung tissues exhibit interstitial collagen accumulation in the same specimen where there is end-stage honeycomb lung (chronic injury) and active fibrosis (acute injury). The fibroblasts present in the actively proliferating fibrotic foci are responsible for ECM deposition, inflammation, and altered mechanical properties in the fibrotic lesions (Dacic & Yousem, IPF supplement, 2003). An imbalance between ECM component production and degradation due to a decrease in normal cellular apoptosis is a significant problem associated with IPF. Increased ECM remodeling in IPF lungs has also been found to correlate with imbalances in matrix metalloproteinase (MMP) family components, notably an increase in tissue inhibitors of

metalloproteinase (TIMPs) (Ramos et al., 2001). MMPs are responsible for degradation of ECM components. TIMPs, in their active form, are inhibitors of MMP catalytic activity. While the process is largely unknown, this is yet further evidence of the role fibroblasts play in the overproduction of ECM components and expanse of fibrotic foci.

Zhang and colleagues (1994) found an increase in the amount of myofibroblasts in bleomycin-induced fibrotic lungs of mice, establishing the correlation between the amount of actively proliferating myofibroblasts and increased level of disease (Zhang et al., 1994). This evidence helped establish the correlation between concentrations of myofibroblasts in their contractile phase and poorer prognosis in subjects with IPF (King et al., 2001). In normal tissues, myofibroblasts appear transiently and are responsible for the production of new collagen and fibronectin at the leading edge of the healing wound. Myofibroblasts in other forms of organizing pneumonias have been shown to undergo normal apoptosis, whereas those associated with IPF remain. The increase in actively proliferating myofibroblasts, the increase in TIMPS, and the increased production of TGF-β, a known protector against apoptosis, shift focus of IPF away from a disease of abnormal inflammation to one of abnormal apoptosis among the myofibroblasts at the fibrotic foci (Phan, 2002).

Nitric oxide signaling

In the late 1980s, the action of nitric oxide (NO) on relaxation of endothelial cells was elucidated (Palmer et al., 1987). The discovery of NO as a

signaling molecule led to the 1998 Nobel Prize in Physiology and Medicine. Nitric oxide is a free radical gas produced from the conversion of L-arginine, molecular oxygen (O_2), and NADPH substrates to L-citrulline and NADP by a group of proteins known collectively as NO synthases (NOS) (Figure 3). NO synthesis also requires the presence of four additional cofactors (tetrahydrobiopterin (BH_4), FAD, FMN, and iron protoporphyrin IX (heme)), as well as calmodulin. (Knowles & Moncada, 1994) The NO produced by the NO synthases diffuses to nearby smooth-muscle cells and binds to the active site heme of guanylyl cyclase. This stimulates the enzyme to increase production of cyclic guanidine monophosphate (cGMP) and ultimately activate cGMP-dependent kinases (Moncada & Higgs, 1993; Knowles & Moncada, 1994). Three major isoforms constitute the NOS family: - neural NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). These three isoforms are products of different genes, under different regulation, have different localization properties, catalytic properties, and inhibitor sensitivity. It should also be noted that the enzymes differ in their constitutive (eNOS and nNOS) verses inducible (iNOS) expression, as well as their calcium-dependence (eNOS and nNOS) or calcium-independence (iNOS) properties (Alderton et al., 2001). Each NOS exhibits oxygenase and reductase structures in which an N-terminal oxygenase domain containing binding sites for heme, BH_4, and L-arginine is linked by a calmodulin-dependent recognition site for FAD, FMN and NADPH. In all isoforms, only dimeric NOSs are catalytically active.

Nitric oxide plays critical roles in vascular biology, including vessel relaxation and inhibition of platelet aggregation, as well as the regulation of

endothelial cell death. The loss of levels of bioavailable NO is an important biochemical marker of early endothelial disfunction found in cardiovascular diseases such as hypertension and atherosclerosis (Boo & Jo, 2003). Long-term inhibition of NO production in the heart leads to an increase in αSMA-positive myofibroblasts with associated collagen and fibronectin deposition in ischemic lesions (Pessanh et al., 2000). Generally, NO shows anti-fibrotic properties in a number of diseases by lowering the levels of differentiated myofibroblasts, levels of injury-inducing reactive oxygen species, and collagen deposition (Vernet et al., 2002).

The formation of NO from L-arginine and NADH is an important part of nonspecific host immunity but also has the unique property of aiding in the maintenance of cell homeostasis (Mohr et al., 1996). There is significant evidence that NO, either delivered by NO donors or generated by NO synthases, initiates apoptotic cell death. The influence at which NO facilitates this apoptosis cascade is dependent on the relative rates of NO formation, its redox state, and combinations with oxygen, superoxide, and other biomolecules (Brüne et al., 1999). This evidence suggests a role of NO in the appearance, differentiation, and disappearance of myofibroblasts at the wound site (Desmouliere et al., 1995). Zhang and colleagues (1995) reported that pulmonary myofibroblasts are more susceptible to NO-mediated apoptosis than are undifferentiated fibroblasts (Zhang et al., 1995).

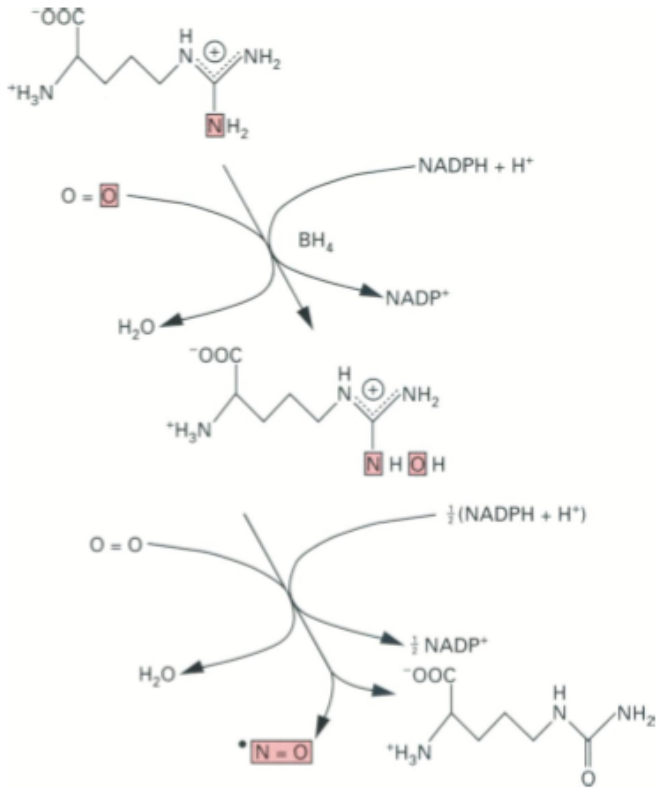

Figure 3: The NO synthase reaction**.** The boxed O and N atoms show the origin of the constituent atoms of NO (Knowles and Moncada, 1994).

NO signaling and pulmonary myofibroblast accumulation

As discussed briefly above, there is ample support for NO-mediated regulation of myofibroblast accumulation and ECM deposition. Unfortunately, few of these studies focus on pulmonary cells. Interestingly, a number of these studies target the eNOS isoform with myofibroblast activity: Ruetten and colleagues (2005) generated knockout mice for each of the three NOS isoforms and demonstrated that eNOS deficient mice have an increase in interstitial fibrosis related to pressure overload-induced left ventricular hypertrophy (Ruetten et al., 2005). IPF is frequently associated with pulmonary hypertension characterized by an increase in mean pulmonary arterial pressure. Vascular

remodeling of the pulmonary artery is the underlying cause of the pulmonary hypertension in IPF patients and treatment with the NO synthase stimulating vascular endothelial growth factor (VEGF) appears to mitigate pulmonary hypertension by reducing endothelial apoptosis, increasing vascularization, and reducing pulmonary arterial pressure. However, VEGF treatment also exacerbates IPF (Farkas et al., 2009). Multiple exposures to bleomycin will induce chronic fibrosis in mouse (and most likely all mammalian) lungs. eNOS knockout mice were observed to maintain a prolonged fibrotic foci after multiple bleomycin exposures, while iNOS knockouts were able to clear the fibrosis as easily as wildtype mice (Chung et al., 2003).

eNOS activation via phosphorylation

Previous work has demonstrated eNOS activation via sheer stress as an effect of blood streaming through vascular tissues (Davies, 1995). There has been ample evidence implicating both an increase or decrease in eNOS catalytic activity due to phosphorylation of serine/threonine residues in the protein (Dimmeler et al., 1999; Fulton et al., 1999; Fleming & Busse, 2003). Two residues in particular, threonine 495 (Thr^{495}) and serine 1177 (Ser^{1177}) (1179 in bovine-derived eNOS) have been shown to have important roles in the regulation of eNOS catalytic activity.

Threonine 495 is located in the calmodulin-binding domain of eNOS and is phosphorylated by a constitutively active AMP-activating kinase or a protein kinase C (PKC). The catalytic activity of eNOS, notably a decrease in production

of NO, is associated with Thr^{495} phosphorylation and is likely explained by the interference with the binding of CaM to the CaM-binding domain of the eNOS protein (Fleming & Busse, 2003). Incubation of endothelial cells with the PKC activator phorbol 12-myristate 13- acetate (PMA) was shown to enhance levels of Thr^{495} (Fleming et al., 2001).

While the activity of eNOS was initially described to be dependent upon the presence of Ca^{2+}, it is now known phosphorylation-induced activity changes can be accomplished without the direct increase or decrease of Ca^{2+} levels (Ayajiki et al., 1996). Fluid shear stress generated by the viscous drag of blood flowing over the surface of endothelial cells results in the activation of phosphatidylinositol 3-kinase, which activates Akt and protein kinase A to phosphorylate eNOS on Ser^{1177} and results in an increase in eNOS activity (Dimmeler et al., 1999).

It has been observed that the phosphorylation of eNOS at one site may affect the phosphorylation at other sites (Bauer et al., 2003). Another serine residue, Ser^{635}, is a frequently observed phosphorylated residue correlating with an increase in eNOS activity and often co-phosphorylated with Ser^{1179}. Investigation using double phospho-mutants of eNOS where both Ser^{1179} and Ser^{635} were mutated to alanine or aspartic acid showed both sites contribute to the regulation of eNOS activity, although phosphorylation of Ser^{635} appears to have greater influence on activity than Ser^{1179} (Boo et al., 2006).

eNOS localization and activity

eNOS localization and activity are correlated in many cells types. In cultured endothelial cells and intact blood vessels, eNOS is found primarily in the caveolae associated with the cell membrane with activity levels correlating with translocation to specific intracellular targets (Garçia-Cardeña et al., 1996). In nephritic tissues, an increase in luminal blood flow in the ascending limb of the loop of Henle, responsible for increasing urine concentration in mammals, induced translocation of eNOS from primarily diffused throughout the cell to the apical membrane. An increase in luminal flow was also observed to increase the amount of phosphorylated S^{1179} eNOS, coinciding with an increase in catalytic activity (Ortiz et al., 2004).

eNOS localization and expression has also been found to be altered in neoplastic tissues. Increased eNOS staining intensity and decreased staining frequency was found to correlate with decreased chance of disease-free survival, and increased cytoplasmic eNOS localization correlated with increased myometrial invasion (an indication of invasive carcinoma) in a study of 50 cases of endometrial carcinoma and normal endometrium (Bentz et al., 1997). A similar correlation between eNOS localization and breast carcinomas (Thomsen et al., 1995) further implicates the important role NO plays in carcinogenesis.

It is likely that numerous events implicated in the regulation of eNOS activity- Ca^{2+}/CaM binding, protein-protein interactions, phosphorylation events, and subcellular localization, are not necessarily independent factors but coordinated functions ultimately affecting the activity of the enzyme. Subcellular-

targeted eNOS mutants show variable localization properties depending on the mutation generated. For example, wild-type eNOS is found in both the plasma membrane and Golgi complex, while a myristoylation-deficient eNOS mutant diffuses among the cytoplasm of the cells. This mutant is also unable to produce NO, while a plasma membrane-targeted mutant produced NO at higher levels than WT eNOS (Boo et al., 2006).

Changes in levels of intracellular calcium also show a relationship to changes in localization and activity of eNOS. Treatment of human skin-derived mast cells with 1 μM of the calcium ionophore A23187 showed moderate increases in cytoplasmic localization and pronounced increases in nuclear localization when analyzed via immunofluoresence. Further analysis of phospho-specific eNOS in cells treated with A23187 indicated increased levels of phospho- Ser^{1177} eNOS in both the cytoplasmic and nuclear cellular fractions, suggesting that increased Ca^{2+} levels potentiate eNOS phosphorylation and, coordinately, activity (Gilchrest et al., 2004).

Calcium- induced activity changes and activation by PKC

Previous investigations from our lab have focused on myofibroblast differentiation, eNOS translocation and activtiy, and the cell products implicated in the regulation of these events. The purpose of the present study was to use cellular fractionation techniques and immunoblot analysis to test the hypotheses that localization in pulmonary myofibroblasts is dependent on growth conditions,

this localization is Ca^{2+}-dependent, and eNOS phosphorylation by PKC regulates localization and, coordinately, activity.

MATERIALS AND METHODS

Animals

Four female Lewis rats (4-6 weeks old, 75-100 g) were purchased from Charles River Laboratories, Willmington MA. All animals were housed in appropriate biological containment facilities at Western Kentucky University and work was carried out in accordance with guidelines established by the Institutional Animal Care and Use Committee (IACUC) and the American Association for Laboratory Animal Science (AALAS) (http://www.iacuc.org/usa/htm).

Establishment of primary myofibroblast cell line

The four Lewis rats were euthanized in order to establish a primary cell line to carry out subsequent work. Pulmonary fibroblasts were isolated as follows: a tracheal/lung lavage was performed three times with 1x Hank's Balanced Salt Solution (HBSS, Invitrogen). The lungs were then gently perfused twice with saline containing 5 μg/mL heparin. The lungs and heart were dissected out and moved to a sterile dish containing cold HBSS. Each lung was then dissected free and placed in cold digestion media (86% 1x HBSS, 0.1% 10x trypsin, 0.02% DNase, 1 mg/mL collagenase, 0.025% HEPES) where they were minced. Each minced lung was allowed to incubate at 37˚C+5% CO_2 for 30 minutes, and then was placed in a conical vial and vortexed vigorously for a few seconds. Dulbecco's Modified Eagles Media (DMEM, Invitrogen) supplemented with 10%

(v/v) fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) was added to each tube to halt the digestion. Tissues were moved to a 100 mm tissue culture dish and macerated with a sterile pipette tip and transferred back to a 50 mL conical tube. Contents of each tube was combined and centrifuged three times at 1200 RPM for 10 minutes. Between each centrifugation, pellets were washed with wash media (DMEM, 5% BCS, Pen/Strep). Cells were then counted using a hemacytometer and diluted accordingly for plating at a concentration of 1-2 x 10^5 cells/100 mm plate. Media was changed every morning for three to four days following prep to eliminate red blood cell contamination. After one week, the cells were passaged 1:3.

Cryopreservation of primary myofibroblasts

Cells were grown in DMEM +10% FBS until they reached a concentration of 1-5 x 10^6 cells/mL. Cells were then resuspended in 1 mL cryovials of freezing media (90% FBS + 10% DMSO) at desired concentration and immediately placed in -80˚C freezer. The vials were transferred to N_2 after 24-48 hours.

Myofibroblast culturing

One 1 mL tube containing primary myofibroblasts was removed from N_2 and quickly thawed in a 37˚C water bath for 2-3 minutes. Cells were resuspended 1:10 in normal growth media (DMEM/10%FBS) and gently pipetted onto a 100 mm dish. Cells were grown to ~70-75% confluence in 37˚C+5% CO_2

incubator, then passaged 1:4 to obtain desired concentration of cells for experiments. Only cells between passages 2-5 were used for all experiments.

Myofibroblast harvesting and assay

Media was aspirated from each plate containing cells at appropriate confluence and 1 mL 0.05% trypsin was added and each plate is allowed to incubate for 30 minutes in 37˚C+5% CO_2 incubator enzymatically loosen cells from the culture dishes. Cells were then washed sterile phosphate-buffered saline (PBS) and transferred to eppendorf tube, where they were pelleted via centrifugation and resupended in 1x lysis buffer (10 mM NaCl, 10 mM Tris-HCL (pH=7.6), 3 mM $MgCl_2$, 0.5% Tween-20) supplemented with protease inhibitor tablet (Roche). Cells were lysed via sonication (3x pulses, 10 seconds, 60% amplitude). Protein concentration was obtained via DC protein assay kit (Bio-Rad) or A280 absorbance using a Thermo Nanodrop ND-100 Spectrophotometer (Stoscheck, 1990).

Nuclear/Cytoplasmic isolation of cell lysates

Fibroblasts were harvested in 5 mL sterile PBS and pelleted via centrifugation at 2,000 RPM for five minutes at 4°C. Cell lysates were then resuspended in 1x lysis buffer and allowed to incubate at room temperature for two minutes, then on ice for 10 minutes. Ten percent NP-40 (Sigma) was added to the volume of lysates to a final concentration of 1% (v/v) and the solution was then passed through a 20-gauge needle 2-3 times. One molar $MgCl_2$ was added

to a final concentration of 5 mM and the nuclei were pelleted by centrifugation at 600g for 5 minutes at 4°C. The cytoplasm-containing supernatant was collected and centrifuged at 12,000g for 5 minutes at 4°C to ensure no nuclear carryover. The remaining pellets comprising the nuclear fraction were washed with 300 μl 1x lysis buffer containing 5 mM $MgCl_2$. This wash sequence was repeated 2-3 times, discarding the supernatant each time. The nuclei were lysed by adding another 300 μl of 1X lysis buffer containing 0.3% sodium dodecyl sulfate (SDS) to the final wash pellet and vortexing. Nuclear DNA was sheared via sonication on ice (10 seconds at 50% amplitude). Cytoplasmic and nuclear fractions were then assayed, aliquoted into working stocks, and stored at -80°C. Adequate fractionation was confirmed by western blot (see below) by probing with a proliferating cell nuclear antigen (PCNA) antibody. PCNA is a 35 kDa protein that accumulates in the nuclei of dividing and transformed cells (Mathews et al., 1984) and the presence/absence of which confirms successful nuclear/cytoplasmic fractionation.

SDS-PAGE and Western Blotting

Western blots were carried out using standard methods (Towbin et al., 1979; Renart et al., 1979) with optimizations for myofibroblast whole-cell lysates. Briefly, cell lysates were boiled and proteins were separated using SDS-PAGE with precast 10% polyacrylamide gels (Bio-Rad) suspended in 5x tris/glycine (125 mM Tris-HCL, 1 M Glycine, 0.5% (w/v) SDS) running buffer. Each lane was loaded with 25 μL of protein extract, and subsequent analyses were performed

by normalizing each blot to the concentration of soluble protein loaded. Purified eNOS (Calbiochem) was used as the positive control for each gel/blot. The gels were transferred to polyvinyldene fluoride membranes (Fisher) using a semi-dry transfer apparatus with optimal transfer at 15 V for 30 minutes using a tris/glycine/methanol transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol). Each membrane was then stained with 0.1% (w/v) Ponceau-S stain to ensure successful transfer. Membranes were then blocked overnight in 5% nonfat dry milk/PBS at room temperature. After blocking, membranes were washed with 1% milk/PBS/0.1% Tween-20 and primary antibody was added at appropriate working dilution, using 1% milk/PBS/0.1% T-20 as diluents. Primary antibodies were diluted follows:

- PCNA monoclonal (Santa Cruz) 1:200
- native eNOS polyclonal (BD Biosciences) 1:200
- phospho-eNOS Ser1177 polyclonal (Santa Cruz) 1:200
- phospho-eNOS Thr495 polyclonal (Santa Cruz) 1:200

Primary antibody was allowed to incubate on the membrane for 1-2 hours at room temperature or overnight at 4˚C. All subsequent washes and secondary antibody dilution was carried out with 1%milk/PBS/0.1% T-20. Horseradish peroxidase-conjugated secondary antibody (Southern Biotech) was allowed to incubate at appropriate dilution on blot for 30 minutes at room temperature. Visualization of membrane(s) was accomplished using ECL chemiluminescent detection (GE Healthcare) and the Alpha-Innotech Fluorochem HD2 Gel Imaging System.

Densitometry analysis

Quantitative analysis of the western blots was performed using densitometry analysis of the pixel densities of each visible band after chemiluminescent staining. Initially, the local background of the band was subtracted from the pixel density value, and then normalized to the protein concentration loaded to give relative density.

Immunofluorescence Microscopy

Fibroblasts were trypsinized from a 100 mm dish when they reached ~70 -80% confluence and seeded onto 24-well plates containing one round microscopic cover slip per well at a density of 4-8 x 10^5 cells/500 μl per well. Cells were then allowed to incubate in CO_2 incubator for 4-6 hours to adhere to cover slips, or 24 hours if a higher confluence was desired. After incubation, and if applicable, media was aspirated and replaced with fresh media containing appropriate treatments. After the 24 hour treatment, cover slips were washed with sterile PBS and fixed for 5 minutes using an ice cold mixture of 70% methanol + 30% acetone. After fixation, cells were washed with PBS and blocked overnight using 10% goat serum. The next day, the coverslips were washed four times with PBS to remove excess blocking serum. eNOS polyclonal antibody (BD Biosciences) was diluted 1:50 in 1% goat serum and incubated for one hour at room temperature. After primary incubation, cells were washed three times each with 1% milk/PBS/0.1% T-20. An IgG secondary antibody (Santa Cruz)

conjugated to a Texas Red fluorophore was added at a 1:1000 dilution and the cover slips were incubated for 30 minutes at room temperature. The washing step was then repeated and cells were mounted on microscope slides using Prolong Antifade (Invitrogen) and allowed to dry overnight at room temperature. Low power images (20x objective) were visualized using the differential interference contrast and DAPI filters of a Zeiss Axioplan-2 epifluorescent microscope. Photographs were then captured using a high power (40x objective) oil-immersion filter and an AxioCam MRm camera and associated software.

RESULTS

eNOS localization is dependent upon cellular differentiation

Primary lung fibroblasts were isolated and cultured from Lewis rats and used in all subsequent experiments. The cultured cells were grown under normal growth conditions (DMEM media supplemented with 10% FBS), and then serum starved for 24 hours to differentiate the cells. For simplification and consistency, all cells grown under normal conditions will be referred to as protomyofibroblasts, while those grown under serum-starvation conditions will be referred to as myofibroblasts. All cells were between passages two and five when used. In order to investigate eNOS localization under both normal and differentiated conditions, fixed cells were probed with an anti-eNOS primary antibody and Marina Blue-conjugated secondary antibody. As shown below in Figure 4A and 4B, eNOS is localized primarily in the nucleus of protomyofibroblasts yet in myofibroblasts eNOS is expressed in the perinuclear/cytoplasmic region of the cells (Figure 4C). Figure 4D are cells grown on coverslips and stained with the secondary antibody only.

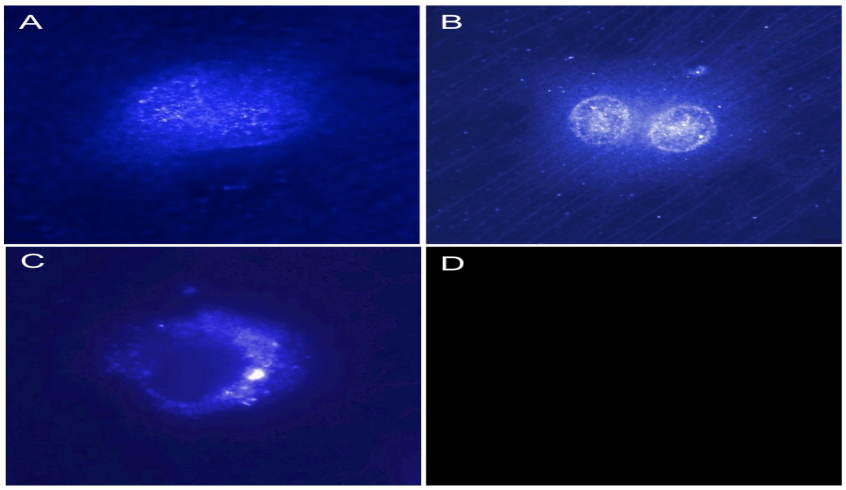

Figure 4: eNOS localization is dependent upon cellular differentiation. Pulmonary myofibroblasts were grown in DMEM supplemented with 10% FBS (A & B) or DMEM with no FBS (C)‡ and then probed with an anti-eNOS primary antibody and a Marina Blue-conjugated secondary antibody. D: Cells stained with secondary antibody alone.

‡It has previously been shown that serum starvation results in a protomyofibroblast to myofibroblast transition characterized by skeletal protein expression (Rice & Leinwand, 2002).

eNOS localization and activity are correlated

Based on the observation that eNOS localization changes in response to cell differentiation, we hypothesized that eNOS translocation would also correlate with the catalytic synthesis of NO in either the nucleus or cytoplasm of the cell. Intracellular levels of NO are qualitatively detectable by immunostaining fixed cells with 4,5-diaminofluorescein-2 diacetate (DAF-2DA) (Leikert et al., 2001; Schwendemann et al., 2008). DAF-2DA is a membrane-permeable fluorescein chromophore that is hydrolyzed to DAF-2 and trapped within the cell where it binds to NO and allows visualization by fluorescence microscopy. As shown in

Figure 5A, treating protomyofibroblasts with 10 μM DAF-2DA stain (Sigma) reveals NO synthesis to be localized primarily to the nucleus, while treated myofibroblasts (Figure 5B) display NO in the perinuclear region of the cell (experiments performed by Ms. Naomi Rowland). Previous data has suggested that eNOS is regulated by multiple mechanisms, including Ca^{2+}/calmodulin binding (Boo et al., 2006). Therefore to investigate the role of Ca^{2+} on eNOS activity in the cells, both protomyofibroblasts and myofibroblasts were also treated with 1 mM of the calcium chelator EGTA. As shown in Figure 5 as well, intracellular Ca^{2+} does influence eNOS activity and localization; most significantly, the chelation of intracellular Ca^{2+} in protomyofibroblasts causes eNOS translocation to mimic that of differentiated myofibroblasts by moving from the nucleus to the perinuclear region (Figure 5C). Catalytic activity of eNOS in myofibroblasts (Figure 5D) does not appear to be affected by EGTA treatment.

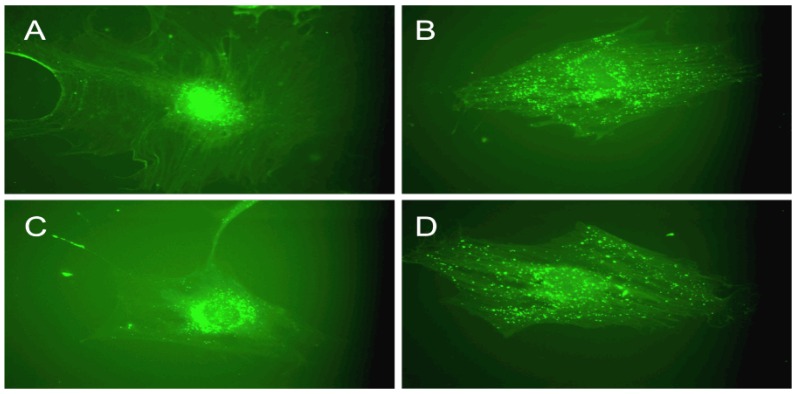

Figure 5: eNOS localization and activity are coordinated A: Protomyofibroblasts stained with DAF-2DA. B: Myofibroblasts stained with DAF-2DA C: Protomyofibroblasts + 1 mM EGTA stained with DAF-2DA D: Myofibroblasts + 1 mM EGTA stained with DAF-2DA.

eNOS is expressed in the nucleus in both protomyofibroblasts and myofibroblasts

In order to more thoroughly quantify eNOS localization and eventually activity, fractionation and immunoblot analysis was used in cells grown under normal and differentiated conditions. Initially, successful fractionation into nuclear and cytoplasmic components was confirmed by probing western blots with an antibody directed at PCNA (Figure 6a and 6b). Nuclear fractions show bands around the 30 kDa ladder marking, correlating with the 35 kDa size of PCNA (Figure 6a). Cytoplasmic fractions probed with anti-PCNA antibody show no bands at the 35 kDa region, confirming cells were adequately separated into nuclear and cytoplasmic fractions.

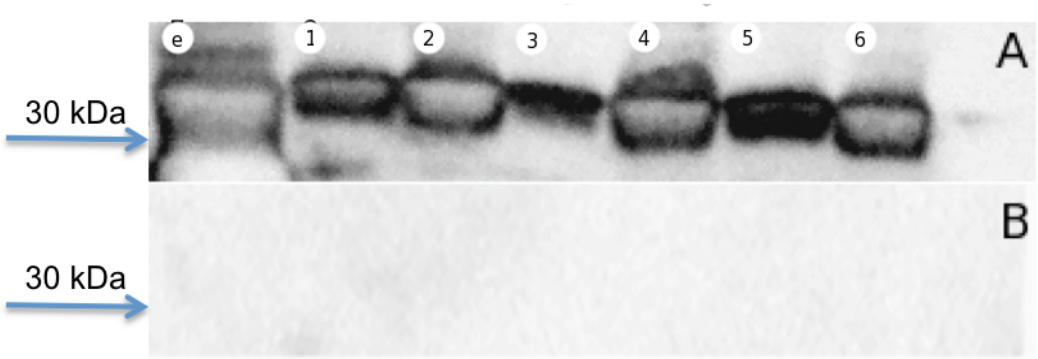

Figure 6: Cellular fractionation is confirmed by presence of PCNA in the nucleus A: (Nuclear fractions) B: (cytoplasmic fractions): Cytoplasmic fractions: Lane e: purified eNOS; Lane 1: Myofibroblasts- untreated; Lane 2: Protomyofibroblasts- untreated; Lane 3: Myofibroblasts- + PMA; Lane 4: Protomyofibroblasts + PMA; Lane 5: Myofibroblasts- + PMA & EGTA; Lane 6: Protomyofibroblasts + PMA & EGTA

Following confirmation of successful fractionation, both nuclear and cytoplasmic extracts were probed with an anti-eNOS polyclonal antibody. As shown in Figure 7 lanes one and two, protomyofibroblasts and myofibroblasts express eNOS in the nucleus. Densitometry analysis (Figure 8), followed by normalization to protein concentration, suggests that nuclear eNOS expression appears higher in differentiated myofibroblasts than protomyofibroblasts, although the difference could not be statistically confirmed. Results are in agreement with previous experiments showing nuclear staining in myofibroblasts, and to a less extent protomyofibroblasts, although no cytoplasmic eNOS staining

was observed for any sample tested (data not shown) when expression levels were quantified by densitometry.

Previous investigations in our lab have shown the expression and activity of eNOS can be regulated by increasing or decreasing the levels of intracellular Ca^{2+}, although it isn't clear if Ca^{2+} is acting directly on eNOS or upstream through the activation of Ca^{2+}-regulated signaling pathways (unpublished results). Indeed, it appears the expression and activity of eNOS can be regulated independently of Ca^{2+} as well, via the phosphorylation of Ser^{1177} (Fleming et al., 2001). Moreover, Ca^{2+}-dependent, PKC agonist-induced activation of eNOS, resulting in the phosphorylation of Thr^{495} in the CaM-binding domain of the protein, has also been reported to downregulate expression and catalytic activity of the protein (Chen et al., 1999).

To further investigate the role of upstream Ca^{2+} signaling pathways in regulating eNOS localization, cells were treated with 10 μM of the PKC activator PMA. Additionally as a control, 150 μM EGTA was added to the cells treated with PMA to mitigate the effects of the agonist. In Figure 7, lanes three and four show myofibroblasts and protomyofibroblasts, respectively, treated with PMA. Lanes five and six show myofibroblasts and protomyofibroblasts treated with PMA and EGTA.

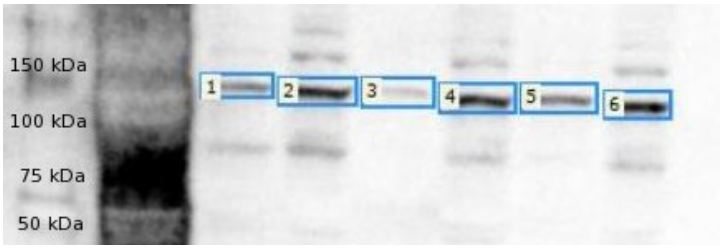

Figure 7: eNOS expression is differentially regulated by Ca^{2+} in protomyofibroblasts versus myofibroblasts. Western blot showing nuclear fractions of treated and untreated cells. Lane 1: Myofibroblasts, no treatment; Lane 2: Protomyofibroblasts, no treatment; Lane 3: Myofibroblasts treated with 10 μM PMA; Lane 4: Protomyofibroblasts treated with 10 μM PMA; Lane 5: Myofibroblasts treated with 10 μM PMA and 150 μM EGTA; Lane 6: Protomyofibroblasts treated with 10 μM PMA and 150 μM EGTA. This blot represents the results of one experiment.

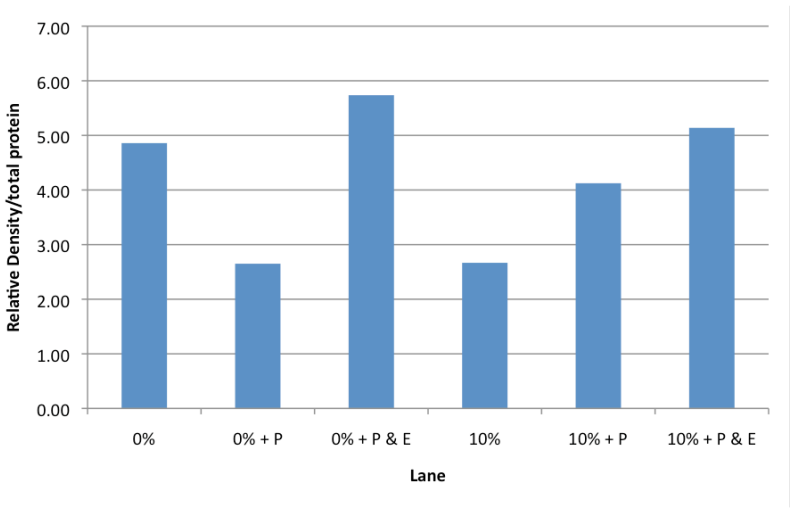

Figure 8: Densitometry analysis of Figure 7. Pixel densities normalized to local background of each boxed lane (Figure 7) and amount of protein loaded per well, grouped according to treatment. 1- Myofibroblasts, no treatment; 2- Myofibroblasts + PMA; 3- Myofibroblasts + PMA & EGTA; 4- Protomyofibroblasts, no treatment; 5- Protomyofibroblasts + PMA; 6- Protomyofibroblasts + PMA & EGTA

Results indicate that when differentiated myofibroblasts are treated with PMA, eNOS expression decreases (Figures 7 and 8). Chelation of Ca^{2+} results in the return of normal eNOS expression levels. Protomyofibroblasts do not respond the same way to PKC activation. eNOS expression is slightly increased in response to PMA treatment in these cells

It is reported that eNOS becomes less active if phosphorylated on Thr^{495} by PKC (Chen et al., 1999), as Thr^{495} phosphorylation may interfere with CaM binding to the enzyme at low Ca^{2+} concentrations. After probing our previous

immunoblotted cell fractions with native eNOS the membranes were stripped of their antibodies and a polyclonal enzyme specific for phospho-eNOS (Thr^{495}) was used to analyze whether or not this residue is phosphorylated in PMA-treated pulmonary myofibroblasts. Results indicate that Thr^{495} is not phosphorylated in either the cytoplasmic or nuclear fractions in either protomyofibroblasts or myofibroblasts. Therefore this particular residue is not implicated in the attenutation of activity or change in expression of the eNOS in our cells.It is possible the polyclonal antibody was not specific to this residue, as it differs in its primary sequence position among mammals expressing the enzyme (ex: bovine eNOS is phosphorylated on Thr^{495}, while human eNOS is phosphorylated on Thr^{497}). Phosphorylation on Ser^{116} has been demonstrated to be inhibited by the PKC inhibitor calphostin (Kou et al., 2002), suggesting other residues are regulated by PKC activation and warrant further investigation.

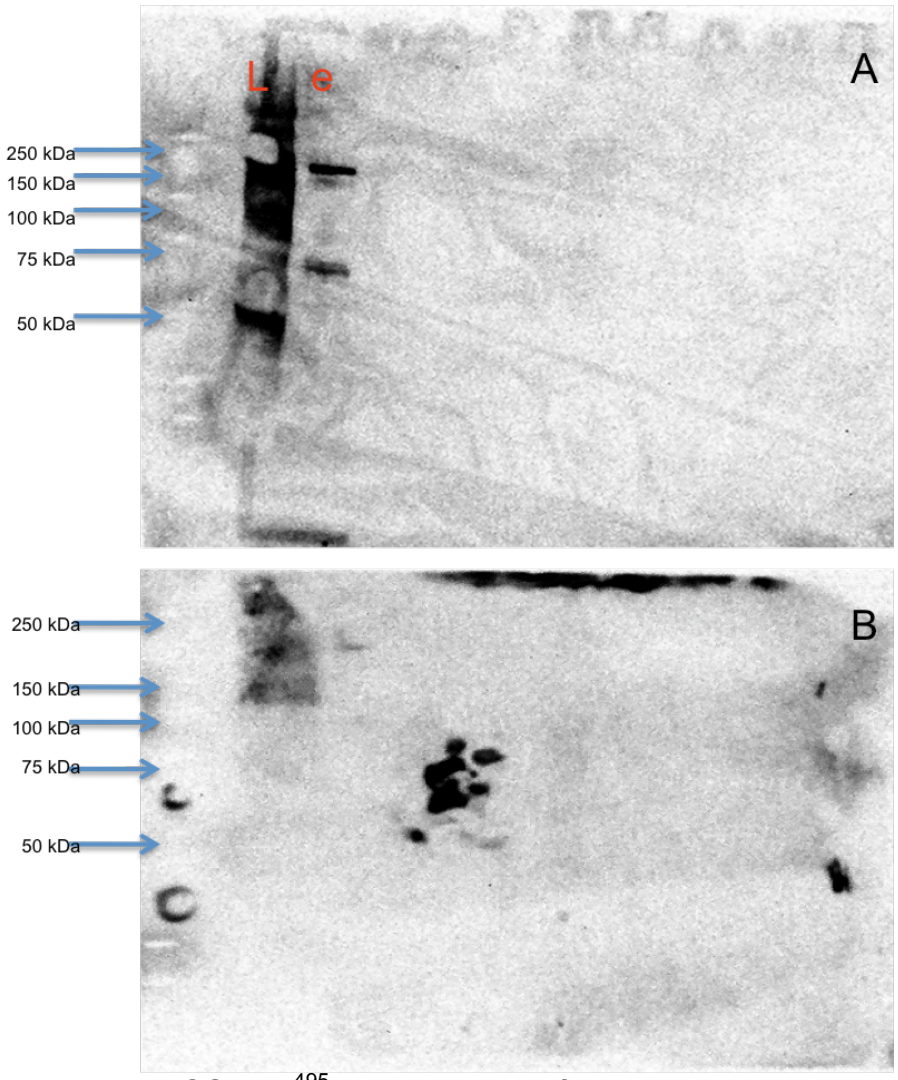

Figure 9: Phospho-eNOS (Thr^{495}) immunoblots of nuclear and cytoplasmic fractions. Nuclear fractions (Figure 9A) reprobed with anti-phosphorylated eNOS (Thr^{495}). “L” indicates the ladder band and “e” indicates the purified eNOS used as a control. Figure 9B indicates cytoplasmic fractions.

DISCUSSION

In recent years, the theory of IPF as a disease consisting predominantly of chronic and uncontrolled inflammation has largely been dismissed as being inaccurate. While many interstitial lung diseases include an identifiable initial inflammatory response to a damaging agent, many patients heal or show vast improvement with anti-inflammatory therapies (Selman & Pardo, 2002). The evolution of the initial inflammatory response to the fibroproliferative pathology characteristic of IPF is largely indistinguishable upon lung biopsy to that of other interstitial lung diseases. Moreover, there is little evidence to suggest true IPF begins with an inflammatory response, or support for the theory that inflammation is prominent in the early phases of IPF (Bjoraker et al., 1998). Indeed, the lack of long-term beneficial response to anti-inflammatory drugs, corticosteroids, and immunosuppressive agents among patients suggests the mechanism behind the onset and fibroproliferative nature of IPF lies in a realm science just beginning to to be understood.

The focus of current research into IPF has generally centered on it as being a disease of fibroproliferation preceded by alveolar cell activation (Selman & Pardo, 2002). Both of these factors have been shown to trigger a number of chemokines/growth factors that induce fibroblast migration and proliferation, and differentiation among myofibroblasts, and subsequent accumulation of ECM (Ramos-Nino et al., 2003: IPF Supplement). It's important to recognize that the potential inflammation and fibroproliferation seen in IPF are two

pathophysiologically distinct pathways independent of each other. Novel treatments and anti-fibrotic therapies are currently in various stages of preclinical/clinical development (de Andrade & Thannickal, 2009) and take aim at the underlying fibroproliferation that is now known to be the hallmark of the disease.

A consistent finding from the various studies into the cell biology, growth-factor/cytokine signaling, animal models, and human models of IPF is the accumulation of the aggregates of myofibroblasts in the fibroblastic foci of the tissue (Thannickal and Horowitz, 2006). Ample evidence exists showing the development of apoptosis-resistant phenotypes of myofibroblasts are generated during fibroblast/myofibroblast differentiation in disease models of IPF, with a correlation among the extent of the fibrotic foci and increased rates of mortality.

Nitric oxide, generated by NOS, plays a substantial role as a signaling molecule involved in physiological processes in the airway epithelium (Gaston et al., 1994; Barnes, 1995). The endothelial isoform of NOS (eNOS) has been demonstrated to be a key enzyme in endothelial tissue homeostasis, angiogenesis, and remodeling. In the last few years, and in this present study, the goal of our research lab has been to characterize and investigate the factors regulating eNOS (as well as iNOS) expression and activity, including post-translational modifications of the enzyme, protein-protein interactions, cofactors, Ca^{2+}/CaM influences, and phosphorylation.

There has been a large amount of research into the biochemical properties of eNOS in a number of endothelial cell types, namely bovine aortic

endothelial cells. Alternatively, there is little research into the enzyme's expression in mesenchymal cells such as pulmonary myofibroblasts, the focus of this thesis. Indeed, a PubMed search of "nitric oxide" and "pulmonary myofibroblast" yields only nine results, while one of "nitric oxide" "bovine aortic endothelial cell" yields 174.

Nitric oxide has been shown to attenuate the epithelial-mesenchymal transition induced by TGF-β in alveolar epithelial cells both *in vivo* and *in vitro*. This feature is also seen in pulmonary tissue biopsies of those patients with lung fibrosis secondary to myofibroblast-mediated excessive ECM deposition (Vyas-Read et al., 2006). Additionally, inhibition of NO synthesis has been shown to increase accumulation of myofibroblasts and subsequent collagen deposition (Pessanh et al., 2000).

This study presents a potential mechanism by which nitric oxide influences myofibroblast accumulation by investigating the role of eNOS in the pulmonary myofibroblast. eNOS knockout mice experience prolonged pulmonary fibrosis in response to the profibrotic agent bleomycin, suggesting a correlation between eNOS expression and myofibroblast regulation and/or apoptosis. We have demonstrated eNOS is the primary NOS isoform expressed in pulmonary myofibroblasts and the enzyme's localization is dependent upon growth conditions. Immunocytochemistry of prtotmyofibroblasts grown under normal (10% FBS) conditions predominately show eNOS expression confined to the nucleus, with little cytosolic staining. Myofibroblasts grown under serum-starved conditions (0% FBS) show an increase in α-SMA and MyHC expression and

mimic *in vivo* differentiated conditions (Rice and Leinwand, 2003). eNOS is translocated from the nuclear to cytoplasmic regions under these highly differentiated conditions (see Figure 4), although nuclear staining is still observed, as shown in figure 7.

In epithelial cells, eNOS has been shown to translocate from the caveolae to intracellular sites in a Ca^{2+}- dependent manner (Goetz et al., 1999). I have shown this too, is the case for pulmonary myofibroblasts. Removal of Ca^{2+} from cells by the chelator EGTA mimics a differentiated state, with eNOS expression largely confined to the perinuclear region. Previous research indicating increases in cytoplasmic Ca^{2+} levels activates CaM, which binds to the canonical CaM-binding domain in eNOS to promote the alignment of the oxygenase and reductase domains of the enzyme, leading to an increase in catalytic activity (Sessa, 2004; Fleming et al., 2001). My data shows pulmonary myofibroblasts respond oppositely to Ca^{2+} presence, with a decrease in basal catalytic activity upon by the removal of Ca^{2+}.

There is ample evidence that regulation of eNOS localization, and, coordinately, activity is regulated via phosphorylation/dephosphorylation on multiple serine, tyrosine, and threonine residues by various protein kinases and phosphatases (Dimmeler et al., 1999; Gallis et al., 1999; Michell et al., 2001). Neuronal NOS (nNOS) was the first NOS isoform to be identified as phosphorylated at distinct sites by PKC, and PKC-dependent phosphorylation decreased the level of enzyme activity (Bredt et al., 1992). We investigated the role of PKC-dependent phosphorylation on eNOS localization and activity by

treating cells with the known PKC activator PMA and immunoblotting fractionated cell lysates for native eNOS and phospho-eNOS (Thr^{495}). PKC activation by PMA has previously been shown to inhibit eNOS activity in cultured cells (Davda et al., 1994; Hirata et al., 1995). In Figure 9, we show that myofibroblasts treated with PMA do indeed synthesize lower than basal levels of eNOS, with the additions of EGTA mitigating this effect. Protomyofibroblasts do not appear to be affected by PMA treatment in the same way showing slight increases in expression. By DAF-2DA staining, we have been also able to show qualitatively that NO is also present in the same cellular regions as eNOS. This work is in agreement with recent work in our lab by Dr. Bethel Sharma who has able to quantify NO activity in our cells grown under various conditions. NO production was measured amperometrically using an NO electrode (Innovative Instruments, Inc) and normalized to total cell count. Treatment with 10 μM PMA was found to lower NO production to near basal levels of differentiated cells. Addition of EGTA to the PMA-treated differentiated cells restored NO production, providing confirmation that phosphorylation of eNOS by PKC is associated with a reduction in its catalytic activity in pulmonary myofibroblasts. Thus, the changes in eNOS expression in response to PKC activation (Figure 8) correlate with changes in the catalytic activity of the protein (Figure 10). These finding are in accordance with earlier data showing eNOS is a PKC substrate and PKC-mediated phosphorylation mitigates eNOS activity (Tsukahara et al., 1993; Davda et al., 1994., Hirata et al., 1995; Fleming et al., 2001).

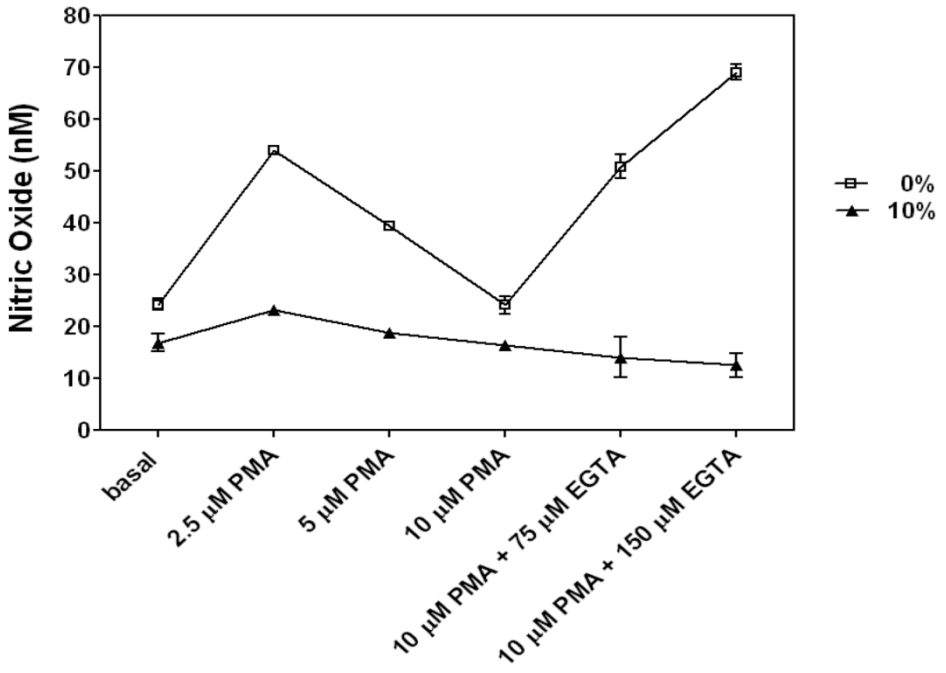

Figure 10: PKC-induced phosphorylation coordinates with activity

These past and present studies have aided in analyzing the role nitric oxide plays as a signaling molecule in the pulmonary myofibroblast, a cell model with little background work, but with important research and clinical implications. With IPF being a disease of uncontrolled fibroproliferation, the possibility of using agents that alter collagen synthesis or mitigate other fibrotic responses are current focuses of first-line treatment for those unresponsive to conventional therapies. With its ability to inhibit microtubule polymerization, colchicine has been investigated *in vitro* and in animal models and demonstrated an ability to suppress the release of macrophage-derived growth factor and fibronectin by cultured alveolar macrophages from patients with sarcoidosis and IPF (Rennard et al., 1988) Treatment with interferon-γ is another novel anti-fibrotic with potential benefits to those with IPF. Ziesche and colleagues have

demonstrated interferon-γ inhibits the proliferation of lung fibrosis in a dose-dependent manner and reduces the synthesis of protein in fibroblasts (Ziesche et al., 1999). Future studies are needed to gain insight into the role nitric oxide plays in the fibroproliferative properties of this cell as implicated in IPF. With the number of existing NO donating or inhibiting pharmaceuticals, the NO pathway could yield yet more treatment options for a disease that, thus far, has tragic consequences.

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