African Journal of Pharmacology and Therapeutics Vol. 2 No. 1 Pages 1-8, 2013
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Research Article

Transcriptional regulation of Rat Endothelial Nitric Oxide Promoter in Pulmonary Myofibroblasts cells and its implications in Pulmonary Fibrosis

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Background: Nitric oxide (NO) levels may exert control on the persistence of pulmonary myofibroblast cells in pulmonary fibrosis.

Objective: This study set out to examine the regulation of NO levels by transcription factors that influence the expression of the endothelial nitric oxide synthase (eNOS3) gene.

Methods: Using a pGL3-Basic vector plasmid DNA, rat and human eNOS3 gene promoters were inserted upstream of a luciferase reporter gene and cloned in competent *E. coli* cells (DH5α). Transfection assays were performed and the cells treated with potential regulators of eNOS3 gene. Promoter activity of eNOS3 gene was assayed using the Dual Luciferase reporter gene assay.

Results: The results indicated that the rat NOS3 promoter was active in the cells, with the human NOS3 promoter showing little or no activity. The results demonstrated that transforming growth factor-β, EGTA and lipopolysaccharide up-regulated transcriptional activity while Phorbol 12-myristate-13-acetate, 23187 and S-nitroso-N-acetylpenicillamine, suppressed eNOS3 transcriptional activity. Treatment with Nw-Nitro-L-arginine methyl ester had no effect on the gene expression.

Discussion: The results of this study demonstrates that high concentrations of NO inhibit NOS3 gene activity, hence an enhanced expression of eNOS in response to pharmacological interventions using some transcriptional factors from these study could provide protection against interstitial pulmonary.

Key words: Nitric oxide, transcription, NOS3 gene promoter, pulmonary fibrosis

Received: November, 2012
Published: March, 2013

1. Introduction

Myofibroblasts in idiopathic pulmonary fibrosis

Myofibroblasts are mesenchymal cells that possess both fibroblast and muscle-like characteristics and function in tissue development, remodeling and wound repair (Gabbiani, 1996). During normal wound healing myofibroblasts undergo apoptosis (Darby et al, 1990; Clark, 1993), but in certain circumstances, these cells persist and continue to secrete extracellular matrix. Persistent myofibroblasts have been implicated in

 ISSN 2303-9841

interstitial fibrosis of the lung characterized by abnormal deposition of extracellular matrix that effaces the normal lung tissue architecture (Phan, 2002). Presence of myofibroblasts in patients with pulmonary fibrosis is amply documented in both lung tissues taken from patients with pulmonary fibrosis as well as in those taken from animal models of the disease (Kuhn and Macdonald, 1991; Pache et al, 1998).

Pulmonary fibrosis, also known as idiopathic pulmonary fibrosis (IPF), is a progressive and largely fatal group of disorders that is quite prevalent worldwide which typically affects patients in their forties and fifties when diagnosed (Lornas et al, 2012). There are five million people worldwide that are affected by this disease and of these more than 40,000 die annually (Selman, et al, 2004). The disease condition has a poor prognosis with a median survival time of 2.5-3.5 years (Barlo et al, 2010). There is, however, limited information regarding the mechanisms of this pathological fibroproliferation. Therefore the mechanism of myofibroblast disappearance is of potential interest since it can provide insight into the basis for its persistence and hence into maintenance or progression of fibrosis.

NO signaling in myofibroblast differentiation

Nitric oxide (NO) produced by endothelial cells via the catalytic action of nitric-oxide synthase (NOS) represents an antifibrotic mechanism in the body. Several studies suggest that NO-mediated signals may be important in regulating myofibroblast phenotypes which, though heterogeneous in different tissues, share many common biochemical characteristics and are valid comparisons (Chung. et al, 2003). In vivo, endothelial NO synthase (eNOS) gene knockout mice demonstrate prolonged pulmonary fibrosis in response to the profibrotic agent bleomycin, suggesting that eNOS operates in down-regulating myofibroblast proliferation and/or mediating apoptosis (Chung et al, 2003). In the heart, long-term inhibition of NO synthesis leads to increased accumulation of myofibroblasts with associated collagen and fibronectin deposition in ischemic lesions causing hypertension and myocardial damage in rats (Pessanh et al, 2000). NO may also play a role in cardiovascular disease. Findings from a randomized, double-blind clinical trial suggest that there may be pharmacogenetic associations between *NOS3* variants and heart failure, stroke and all-cause mortality. (Zhang et al, 2012). NO has been shown to reduce myofibroblast accumulation and collagen deposition (Vernet et al, 2002). These studies support the hypothesis that NO plays a role in mechanisms that down-regulates myofibroblast phenotype expression. A loss of this control may play an important role in development of pulmonary fibrosis. The present study investigated the role of NO in pulmonary fibrosis at eNOS gene activity level.

NOS3 promoter regulation

This study focused on the role of eNOS in myofibroblast function at the promoter level. Promoters such as NOS3 promoter contain specific DNA sequences and response elements which provide a binding site for RNA polymerase and for transcription factors that recruit RNA polymerase (Agullo, 2007). The activity of a gene promoter is regulated by various factors which include effector molecules which either activate or inactivate the gene expression and ultimately the cells (Govers and Rabelink, 2001). This study examined the transcriptional regulation of eNOS gene promoter in pulmonary myofibroblasts and how different regulators that influence promoter activity can be exploited in pharmacological interventions to provide protection against pulmonary fibrosis. Elucidation of the mechanism involved in the genesis of the myofibroblast should provide insight into both pathogenesis of chronic fibrotic diseases and therapeutic strategies for their management and control (Phan, 2012).

2. Methods

2.1 Cloning of rat and human eNOS3 promoters

Rat and human eNOS3 gene promoter were cloned by inserting into pGL3-Basic vector plasmid DNA and pGL2-Basic vector plasmid respectively (Promega Corporation) (Marsden et al, 1993; Li et al, 2008) and introduced into competent *Escherichia. coli* (DH5α) (Takara Bio Incorporation) and grown in liquid LB growth media (Invitrogen Corporation). Inoculated media flasks were incubated at 37 °C (230 rpm for 16 hours). Upon transformation, 200 µl ampicillin (50 mg/ml) was added, transformed *E. coli* cells that took up the plasmid with the selection marker which confers ampicillin resistance were harvested by centrifugation, and pelleted bacteria containing plasmid DNA recovered.

The plasmid DNA was purified using QIA filter midi-prep plasmid purification kit (Qiagen Company) and quantified using a Nano drop spectrophotometer. Both the rat and human NOS3 promoters were cleaved from the plasmid vector using KpnI and XhoI restriction endonucleases (Promega Corporation). 0.7% agarose gel electrophoresis was used to visualize and determine the presence and size of the DNA bands. The pGL3-NOS3 plasmid was sequenced by chain termination method using the Big Dye Terminator v3.1 Cycle sequencer to determine the exact sequence of the promoter as per the manufacturer's protocol (Applied Biosystems Inc.). The function of the DNA sequence was known using National Center for Biotechnology Information (NCBI) (U.S.A) with database sequence search done with Basic Local Alignment Search Tool (BLAST) and vector NTI.

2.2 Cell culture

Rat myofibroblast cells were grown and passaged for 48 hours in growth conditions comprising of 5% carbon dioxide at 37 °C in normal growth media containing Dulbecco's Modified Eagles Medium (DMEM, Gibco 11960), 10% FBS (Fetal Bovine Serum), 100 ug/ml Penicillin and 100 ug/ml streptomycin and 2 mM glutamate and 1.0 M HEPES pH 7.4. Pulmonary myofibroblast cells of 80% confluence between 4-5 passages were used. The cells were harvested in 1% Fetal Bovine Serum (FBS)(differentiating media) and then seeded to 24 well plates at a density of 4 x 10^5 cells/well and incubated for 16 hrs in 37 °C prior to transfection. The seeded cells were transfected with plasmid vectors with promoter inserts

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Figure 1: The NOS3 promoter sequence between KpnI and XhoI restriction sites (underlined and bold).

2.3 Transfections

Rat and Human NOS3 promoters in pGL3 and PGL2-basic plasmid DNA respectively were transfected using a cationic lipid, Lipofectamine 2000 (Invitrogen). The cells were cotransfected with SV 40 Luciferase which is a positive control containing SV40 promoter and enhancer sequences resulting in strong expression of *luc+* and PGL3 Basic which is the negative control lacking the eukaryotic promoter and enhancer sequences. They were also cotransfected with a Renilla Luciferase construct downstream of the Thymidine kinase promoter (RLTK) to normalize activity levels and control for variability in transfection efficiency. Following transfection, cells were allowed to grow for 36-48 hrs at 37 °C in a 5% carbon dioxide incubator. The cells were then harvested and assayed for Luciferase activity using the Dual Luciferase assay reporter gene technique (Promega) recording the relative luminescence of Firefly and Renilla Luciferase activities. Firefly Luciferase activity was normalized to that of Renilla Luciferase activity (relative activity).

Various effectors known to affect myofibroblast activity were used to determine their effects on eNOS3 gene expression in pulmonary myofibroblast cells. Their concentrations were chosen based on the half-life of each molecule as well physiologically relevant concentration that elicits an effect and on published data. 20 µM and 2 µM concentrations of Phorbor-12-myristate-13-acetate (Calbiochem), 10 ng/ml and 5 ng/ml concentrations of Transforming growth factor β) (TGFβ) (Calbiochem) (Desmouliere et al, 1993; Searles, 2006), 10 ng/ml of Lipopolysaccharide (LPS) (Sigma) (Searles, 2006), 1 mM and 10 mM concentrations of Nω-nitro-L-arginine methyl ester (L-NAME) (Sigma-Aldrich) (Huige et al, 2004, Searles, 2006), 0.1 mM and 1 mM concentrations of S-nitroso-N-acetylpenicillamine (S-NAP) (Calbiochem) (Huige et al, 2004), 1 mM and 1 µM concentrations of Ethyl glycol tetra acetate (EGTA) and 1 µM of A23187 (Calbiochem) (Feron et al, 1998) were tested.

2.4 Statistical analysis

In each experiment, control and experimental rat pulmonary myofibroblast cells were matched for cell line, age, seeding density, number of passages and number of days post-confluence to avoid variation in tissue culture factors that may have influenced the measurements of eNOS activity. Results were shown as means ± SE for *n* experiments and mean values were normalized to the control making the values easily comparable to the negative control. Difference in means among different concentrations used was analyzed by one way ANOVA .P-values <0.05 were considered significant.

3. Results

Sequencing results

There were 1018 base pairs between KpnI and XhoI restriction sites corresponding to the rat NOS3 promoter construct in PGL3 and 1.3 kb of human eNOS3 promoter between KpnI and BglII. This was consistent

with the results from the gene bank of vector NTI gene analysis programme (**Figure 1**).

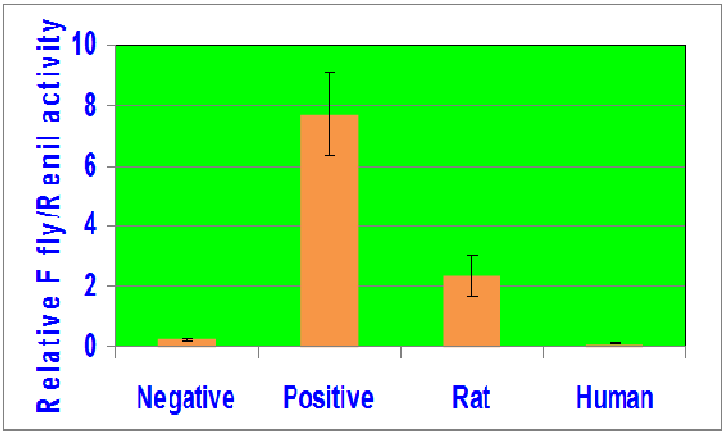

Figure 2: Base line data of the relative Fire fly activity/Renilla activity of PGL3 basic (Negative control), SV 40 Luciferase (Positive control), the Rat-NOS3 promoter and Human NOS3 promoter in rat pulmonary myofibroblast cells.

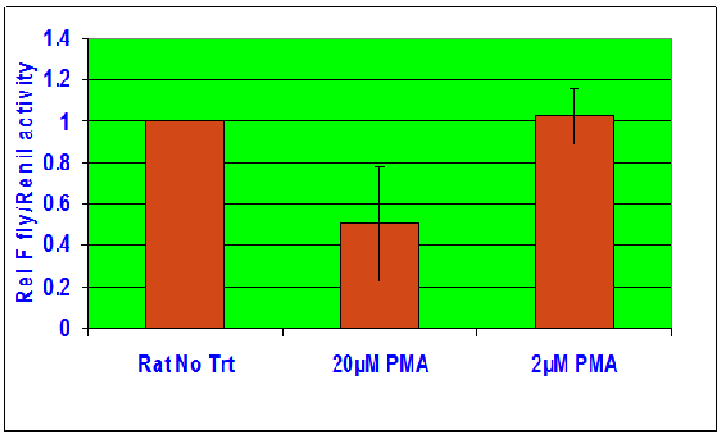

Figure 3: The effect of PMA on relative Rat promoter activity.

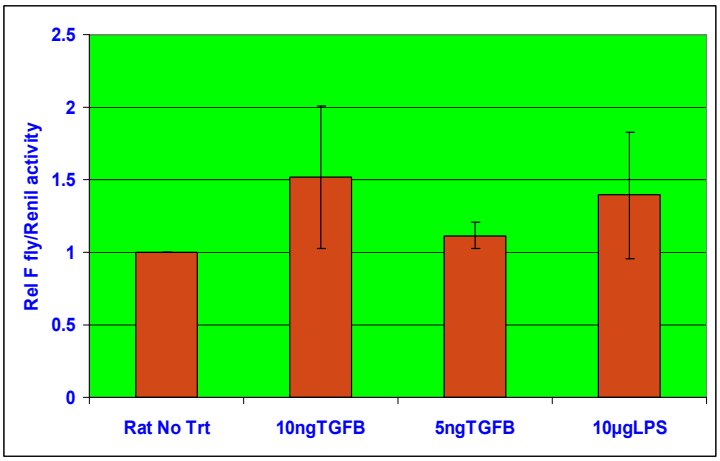

Figure 4: The effect of TGFβ and LPS on relative Rat promoter activity in rat pulmonary myofibroblast cells (mean values were normalized to the control).

Baseline luminescent activity of rat and human NOS3 promoter in pulmonary myofibroblast.

The mean relative activity of the firefly activity/renilla pGL3 basic vector negative control was 0.253 ± 0.0624. The mean relative activity value for the SV 40 Luciferase positive control was 7.728 ± 1.3758. The mean relative activity value for the Rat-NOS3 promoter with a Renilla Luciferase construct was 2.358±0.6744 and the mean relative activity value of Human-NOS3 promoter with a Renilla Luciferase construct was 0.0878±0.0240 (**Figure 2**).

Influence of various effectors on the Rat- NOS3 promoter activity in rat pulmonary myofibroblasts

PMA

The relative Fire fly activity/Renilla activity mean value for 20 µM PMA was 0.50 ± 0.2751; the relative activity mean value for 2µM PMA was 1.03 ± 0.1319. (**Figure 3**)

TGFβ and LPS

The relative Firefly activity/Renilla activity of the negative control rat without treatment mean value was 1.0 ± 0.00; the relative activity mean value for 10 ng TGFβ was 1.52 ± 0.4895 and the relative activity mean value for 5 ng TGFβ was 1.12 ± 0.08651.The relative activity mean value for 10µg LPS was 1.39±0.4351 (**Figure 4**).

L-NAME

The relative Firefly activity/Renilla activity of L-NAME; rat without treatment mean value was 1.0 ± 0.00, the relative activity mean value for 1 mM L-NAME was 1.11 ± 0.1072, the relative activity mean value for 10 mM L-NAME was 1.06 ± 0.16866 (**Figure 5**).

SNAP

The relative activity mean value of 0.1 mM S-NAP was 0.99 ± 0.1477 while the relative activity mean value for 1 mM S-NAP was 0.29 ± 0.0470 (**Figure 6**).

23187(calcium ionophore) and EGTA

The relative Firefly activity/Renilla activity of rat without treatment mean value was 1.0 ± 0.00, the relative activity mean value for 1 µM 23187 was 0.51 ± 0.1256.The relative activity mean value for 1 mM EGTA was 0.94 ± 0.19814 and the relative activity mean value of 1 µM EGTA was 1.23 ± 0.20574. (**Figure 7**)

4. Discussion

This study examined the transcriptional regulation of the eNOS3 gene in pulmonary myofibroblast cells. Information on this gene regulation mechanism may offer potential for therapeutic intervention against pulmonary fibrosis emanating from altered characteristics of myofibroblasts. Studies suggest that inhibition of NO production leads to increased accumulation of myofibroblasts (Vernet et al, 2002) therefore enhancing expression of eNOS using pharmacological interventions could provide protection against fibrosis, specifically interstitial pulmonary

ISSN 2303-9841

fibrosis (IPF). There are several factors that regulate the transcription of eNOS gene such as shear stress (Davis et al, 2004), estrogen and hypoxia. Other factors modulate the stability of its mRNA which includes tumor necrosis factor alpha (TNF-α), LPS and vascular endothelial growth factor (VEGF).

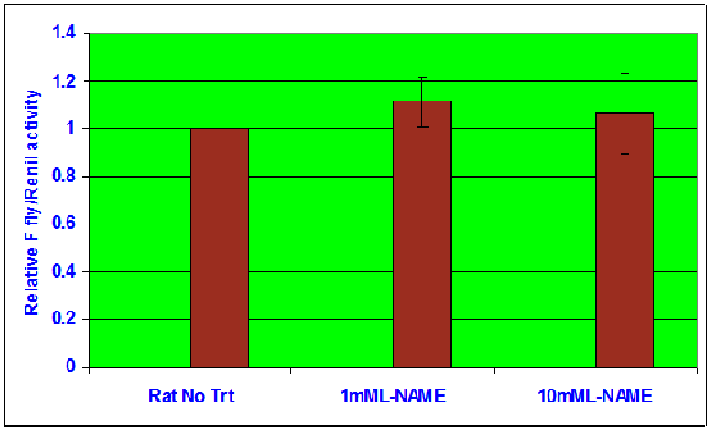

Figure 5: The effect of L-NAME on relative Rat promoter activity in rat pulmonary myofibroblast cells (Mean values were normalized to the control).

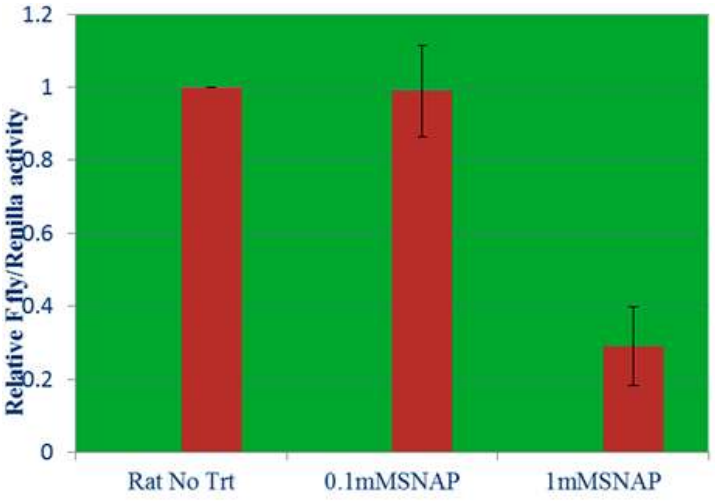

Figure 6: The effect of SNAP on relative Rat promoter activity in rat pulmonary myofibroblast cells. (Mean values were normalized to the control).

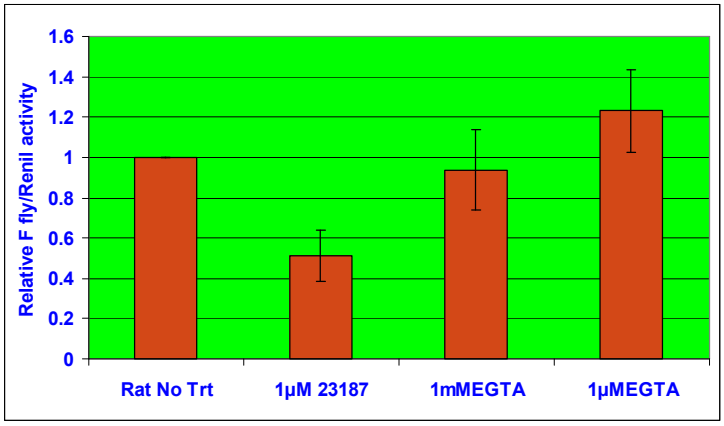

Figure 7: The effect of 23187 and EGTA on relative Rat promoter activity in pulmonary myofibroblast cells (Mean values were normalized to the control).

These results indicate that rat NOS3 promoter was active in rat myofibroblast cells while the human NOS3 promoter was inactive (**Figure 2**).The present study demonstrated that treatment of rat pulmonary myofibroblast cells with increased concentration of Phorbol 12-myristate 13- acetate (PMA) downregulates NOS3 gene transcription.

PMA is a diester of phorbol known to activate the signal transduction enzyme protein kinase C (PKC) and its effects on this molecule result from its structural similarity to one of the natural activators of classic PKC isoforms, diacylglycerol (Agullo, 2007). PKC is a family of enzymes that are involved in controlling the function of other proteins through the phosphorylation of hydroxyl groups of serine and threonine amino acid residues on these proteins. PKC enzymes in turn are activated by signals such as increases in the concentration of diacylglycerol or Ca^{2+}. Hence PKC enzymes play important roles in several signal transduction cascades (Mellor and Parker, 1998; Sellak et al, 2012).

The regulatory domain or the amino-terminus of the PKCs contains several shared sub regions. The C1 domain, present in all of the isoforms of PKC has a binding site for diacylglycerol (DAG) as well as non-hydrolysable, non-physiological analogues called phorbol esters such as PMA. This domain is functional and capable of binding DAG in both conventional and novel isoforms; however, the C1 domain in atypical PKCs is incapable of binding to DAG or phorbol esters. The C2 domain acts as a Ca^{2+} sensor and is present in both conventional and novel isoforms, but functions as a Ca^{2+} sensor only in the conventional. The pseudo substrate region, which is present in all three classes of PKC, is a small sequence of amino acids that mimic a substrate and bind the substrate-binding cavity in the catalytic domain; it lacks critical serine, threonine phosphor-acceptor residues, keeping the enzyme inactive. When Ca^{2+} and DAG are present in sufficient concentrations, they bind to the C2 and C1 domain, respectively, and recruit PKC to the membrane. This interaction with the membrane results in release of the pseudo substrate from the catalytic site and activation of the enzyme (Mellor and Parker, 1998).

Upon activation; protein kinase C enzymes are translocated to the plasma membrane by RACK proteins (membrane-bound receptor for activated protein kinase C proteins). The protein kinase C enzymes are known for their long-term activation: They remain activated after the original activation signal or the Ca^{2+}-wave is gone. This is presumably achieved by the production of diacylglycerol from phosphatidylinositol by a phospholipase; fatty acids may also play a role in long-term activation (Mellor and Parker, 1998).

PMA may be acting through this PKC signal transduction pathway to regulate the transcription of the eNOS3 gene. A multiplicity of functions has been ascribed to PKC. Recurring themes are that PKC is involved in receptor desensitization, in modulating membrane structure events, in mediating immune responses, in regulating cell growth, and in learning and memory and in regulating transcription which is the focus of this study (Mellor and Parker, 1998). These functions are achieved by PKC-mediated

phosphorylation of other proteins and the substrate proteins present for phosphorylation are variable, since protein expression is different between different kinds of cells. Thus, effects of PKC are cell-type specific.

The results of this study demonstrated that high concentrations of PMA (20 μM) increased NOS3 gene expression and the fact that PMA resembles one of the natural activators of classic PKC isoforms, diacylglycerol, we can therefore postulate that it acts in the same cascade through the PKC pathway to down regulate the transcription of NOS3 gene in pulmonary myofibroblasts (Agullo, 200; Hutchinson et al, 2012). This finding can therefore be an important tool in pharmacological intervention investigations.

Moreover, the present study also demonstrates that increasing calcium in the cell decreases eNOS3 promoter activity in rat pulmonary myofibroblast cells by the effects seen by A23187 while chelation of calcium by EGTA enhances expression of NOS3. EGTA is a polyamino carboxylic acid, a chelating agent that is related to the better known EDTA, but with a much higher affinity for calcium than for magnesium ions (Bett et al, 2002).

Calcimycin, which is a calcium ionophore, is a mobile ion-carrier that forms stable complexes with divalent cations (ions with a charge of +2) through the increase of intracellular Ca^{2+} levels in intact cells thus suppressing NOS3 promoter activity (Agullo, 2007). Calcimycin also uncouples oxidative phosphorylation, the process cells use to synthesize ATP which they use for energy, and also inhibits mitochondrial ATPase activity (Agullo, 2007). All NO-synthases require to be bound to the calcium regulatory protein calmodulin for activation. iNOS tightly binds calmodulin even at resting calcium concentrations, and then it is active once it is synthesized. However, constitutive enzymes, eNOS and nNOS, only bind calmodulin when the intracellular calcium concentration increases up to a certain value. Agents that increase intracellular calcium concentration like Calcimycin, either by allowing calcium entrance from the outside or by stimulating calcium mobilization from intracellular stores, can activate these constitutive enzymes.

It is now clear that eNOS is also regulated by pathways that are independent of changes in the intracellular calcium concentration and that eNOS activity is largely dependent on intracellular localization and on phosphorylation at specific amino acids (Agullo, 2007).The study aimed at finding out the effect of chealation of calcium ions or the availability of the calcium ions to the cells in relation to transcriptional regulation of the eNOS3 promoter. The study confirmed that increase of calcium concentration by Calcimycin suppresses eNOS3 promoter activity in rat pulmonary myofibroblast cells and therefore postulates that this may be due to uncoupling of oxidative phosphorylation and inhibition of mitochondrial ATPase activity in these cells by calcium. Removal of calcium by the action of EGTA up regulates the expression of NOS3 gene, this study postulates that this may be acting through the pathway independent of calcium concentration but dependent on eNOS intracellular localization and on its phosphorylation at specific amino acids.

Furthermore, the results also demonstrated that blocking NO production has no effect on the expression of NOS3 gene in pulmonary myofibroblast cells through the effect seen by L-NAME. L-NAME is a potent eNOS selective inhibitor (Rotzinger et al, 1995; Yu et al, 2012). These results therefore suggest that L-NAME does not act through transcriptional mechanism but posttranscriptional through the inhibition of eNOS protein production by suppression of transduction.

However, this study demonstrates that high concentrations of the NO downregulates eNOS expression and therefore we can confirm the hypothesis that NO acts to regulate its own transcription. S-NAP is a NO donor and liberates NO spontaneously without any requirement for enzyme degradation. Recent studies have shown that S-NAP has a retaining ability to produce cyclic-guanosine monophosphate (Shaffer et al, 1992). S-NAP is formed by addition of a nitroso group to a sulfur atom of an amino acid residue a process known as S-nitrosation or S-nitrosylation which is a reversible process and a major form of posttranslational modification of protein (Yang and Loscalzo, 2007).

S-nitrosylated proteins (SNO's) serve to transmit nitric oxide (NO) bioactivity and to regulate protein function through mechanisms analogous to phosphorylation. NO donors target specific amino acids motifs; leading to changes in protein activity, protein interactions, or subcellular location of target proteins. NOS activity leads directly to SNO formation. NOSs are hemoproteins that combine reductase and oxygenase catalytic domains in one monomer to synthesize NO from the terminal nitrogen atom of L-arginine in the presence of NADPH and oxygen. NOSs target specific Cys residues for S-nitrosylation (Gaston et al, 2003). Thiol S-nitrosylation and NO transfer reactions (transnitrosation reactions) are involved in virtually all classes of cell signaling, ranging from regulation of ion channels and G-protein coupled reactions to receptor stimulation and activation of nuclear regulatory protein (Gaston et al, 2003). We postulate that S-NAP may be operating through this pathway possibly targeting the eNOS protein. The present study also demonstrated that Lipopolysaccharide (LPS) upregulate the transcription of eNOS gene probably by modulating the stability of its mRNA.

We have also demonstrated that high concentrations of TGFβ up-regulates the expression of eNOS3 gene. TGF-β is a protein that controls proliferation, cellular differentiation, and other functions in most cells and plays a role in immunity, cancer, heart disease, diabetes, and Marfan syndrome. TGF-β acts as an antiproliferative factor in normal epithelial cells and at early stages of oncogenesis. Some cells secrete TGF-β, and also have receptors for TGF-β. Cancerous cells increase their production of TGF-β, which also acts on surrounding cells (Khalil, 1999). TGF-β induces apoptosis in numerous cell types and postulated to act through the SMAD pathway (Khalil, 1999). SMADs are proteins that modulate the activity of transforming growth factor beta and often in complex with other SMADs/CoSMAD, act as transcription factors that regulate the expression of certain genes. TGF-β may therefore be acting through the SMAD pathway to upregulate the expression of eNOS in pulmonary myofibroblast cells.

 ISSN 2303-9841

The SMAD pathway is the canonical signaling pathway that TGF-β family members signal through. In this pathway, TGF-β dimers bind to a type II receptor which recruits and phosphorylates a type I receptor. The type I receptor then recruits and phosphorylates a receptor regulated SMAD (R-SMAD). SMAD3, an R-SMAD, has been implicated in inducing apoptosis. The R-SMAD then binds to the common SMAD (coSMAD) SMAD4 and forms a heterodimeric complex. This complex then enters the cell nucleus where it acts as a transcription factor for various genes, including those that activate the mitogen-activated protein kinase 8 pathways, which triggers apoptosis (Khalil, 1999)**.** Transcription factors perform this function alone or with other proteins in a complex, by promoting (as an activator), or blocking (as a repressor) the recruitment of RNA polymerase (the enzyme that performs the transcription of genetic information from DNA to RNA) to specific gene.A defining feature of transcription factors is that they contain one or more DNA-binding domains (DBDs), which attach to specific sequences of DNA adjacent to the genes that they regulate. (Roeder, 1996)

The factors and elements investigated in the present study can therefore be manipulated to bring the effects desired in the investigation of a rational therapeutic design. The present study therefore overally concludes that the rat eNOS promoter is active in pulmonary myofibroblasts and the activity of the eNOS promoter appears to be regulated by various mechanisms including NO itself. The suppression of the eNOS promoter by high concentrations of NO suggests that possibly during the inflammatory process of IPF, NO is produced by iNOS resulting in suppression of eNOS activity.

Conflict of Interest declaration

The authors declare no conflict of interest

Acknowledgements

This work was made possible by academic research enhancement award by Western Kentucky University (WKU). The authors wish to acknowledge the biotechnology centre staff at WKU, as well as Prof. Jiang Li of University of Pittsburg, USA for kindly providing the NOS3 rat and human promoters, important components of this work.

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 ISSN 2303-9841