COUNTERREGULATORY ROLES OF TRANSFORMING GROWTH FACTOR (TGF)-β AND ATRIAL NATRIURETIC PEPTIDE (ANP) IN PRESSURE OVERLOAD-INDUCED CARDIAC REMODELING AND FIBROSIS

by

JASON ANTHONY LUCAS

SUZANNE OPARIL (MENTOR)
SUSAN BELLIS
J. EDWIN BLALOCK
YIU-FAI CHEN
GILBERT PERRY
JOANNE MURPHY-ULLRICH

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham, in partial fulfillment of the requirements for the degree of Doctor of Philosophy

BIRMINGHAM, ALABAMA

2009
Pressure overload stress (e.g., hypertension or aortic stenosis) results in excessive cardiac fibrosis, changes in left ventricular (LV) geometry, and disruption of LV contractility, ultimately leading to heart failure. We hypothesize that transforming growth factor-beta (TGF-β), a pro fibrogenic factor, and atrial natriuretic peptide (ANP), an anti fibrogenic factor, interact directly in a counterregulatory manner to balance the production of extracellular matrix (ECM) under pressure overload induced cardiac stress or hypoxia-induced pulmonary vascular adaptation. In this report, we examine structural and functional responses of the intact heart to pressure overload stress in vivo. We then utilize isolated rat pulmonary artery smooth muscle cells (PASMC) and mouse cardiac fibroblasts (CF) to delineate on the cellular level the counterregulatory roles of TGFβ and ANP/cGMP/protein kinase G (PKG) in determining stress responses in vitro.

First, we utilized a DnTGFβRII mouse model to define the contribution of TGFβ signaling to the phenotype of cardiac remodeling and fibrosis in response to pressure overload. We demonstrated that inhibition of TGF-β signaling attenuates pressure overload-induced interstitial non-myocyte proliferation and
collagen deposition with subsequent development of LV dilation and systolic dysfunction.

Second, we demonstrated that ANP has an anti-fibrogenic effect on PASMC treated with TGF-β. ANP and cGMP suppressed the TGF-β stimulated ECM gene expression by interfering with Smad signaling through a protein kinase G (PKG)–dependent mechanism. The most striking finding of this study is that ANP and cGMP inhibit TGF-β1-induced nuclear translocation of pSmad2 and pSmad3, but not the phosphorylation of Smad2 and Smad3 in PASMCs, thus defining a novel molecular mechanism by which ANP signaling intercepts the TGF-β signaling pathway and blocks TGF-β-induced ECM expression.

Finally, we demonstrated a precise site of “molecular merging” of pro-fibrogenic (TGFβ/Smad) and anti-fibrogenic (ANP/cGMP/PKG) pathways in CFs: PKG induced hyper-phosphorylation of Smad3 protein, with resultant failure of nuclear translocation of pSmad3 and consequent inhibition of TGFβ/Smad induced ECM gene expression. Thus, we have demonstrated cross talk between ANP and TGF-β signaling pathways in CFs in vitro such that ANP/cGMP, through a PKG dependent mechanism, block induction of ECM expression by TGF-β1.

Keywords: Transforming growth factor-beta, atrial natruretic factor, cardiac fibroblast, cardiac fibrosis and remodeling, signal transduction
DEDICATION

I would like to dedicate this dissertation to the most important people in my life. First and foremost to my wife, Julie, who has been by my side encouraging and strengthening me since the day I started graduate school. Julie has always listened to my complaints and held my hand when I needed it. To my parents, Ron and Pennie, who have been consistently supportive of all the choices I have made in my life.
ACKNOWLEDGEMENTS

First, I would like to acknowledge God for giving me the strength and courage to undertake several years of training in the sciences. Without his spiritual presence I would have never survived.

I would like to acknowledge several people that without their help and guidance, my Ph.D., would have never come to fruition. I would like to thank my mentors Suzanne Oparil and Yiu-Fai Chen.

I would also like to thank those who have helped me on my research project. Peng Li, my office mate and friend. Li spent countless hours guiding me through various laboratory experiments and techniques even when it would have been much faster to do it himself. Yun Zhang, our animal surgeon, and my friend. Yun spent a lot of time with me caring for the animals and discussing surgery. There were other members of the Oparil laboratory who touched my life and I would like to acknowledge: Daisy Xing, Kaizheng Gong, Fadi Hage, and Faith Foster. Thank you all.
TABLE OF CONTENTS

ABSTRACT ............................................................................................................................. iii
DEDICATION .......................................................................................................................... v
ACKNOWLEDGEMENTS ..................................................................................................... vi
LIST OF TABLES ................................................................................................................... ix
LIST OF FIGURES ................................................................................................................ x
LIST OF ABBREVIATIONS .................................................................................................... xiv
INTRODUCTION .................................................................................................................. 1
   Hypertensive Heart/Lung Disease .................................................................................. 1
   Transforming Growth Factor beta .............................................................................. 3
   Atrial Natriuretic Peptide ............................................................................................. 5
   Signaling Pathway for TGF-β ..................................................................................... 6
   Cardiac Fibroblast and Extracellular matrix proteins ............................................... 8
   Collagen ......................................................................................................................... 9
   Pulmonary Artery Smooth Muscle Cells and the ECM .............................................. 10
   Transgenic Animal Models ......................................................................................... 11
   Project Summary .......................................................................................................... 14

INHIBITION OF TRANSFORMING GROWTH FACTOR (TGF)-β SIGNALING
INDUCES LEFT VENTRICULAR DILATION AND DYSFUNCTION IN THE
PRESSURE OVERLOADED HEART .................................................................................. 16

ANP SIGNALING INHIBITS TGF-β-INDUCED SMAD2 AND SMAD3 NUCLEAR
TRANLOCATION AND EXTRACELLULAR MATRIX EXPRESSION IN RAT
PULMONARY ARTERIAL SMOOTH MUSCLE CELLS ......................................................... 49

ATRIAL NATRIURETIC PEPTIDE INHIBITS TRANSFORMING GROWTH
FACTOR-β-INDUCED SMAD SIGNALING AND MYOFIBROBLAST
TRANSFORMATION IN MOUSE CARDIAC FIBROBLASTS .............................................. 84
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DISCUSSION AND FUTURE STUDIES</td>
<td>116</td>
</tr>
<tr>
<td>GENERAL LIST OF REFERENCES</td>
<td>126</td>
</tr>
<tr>
<td>APPENDIX: INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORMS</td>
<td>135</td>
</tr>
</tbody>
</table>
LIST OF TABLES

INHIBITION OF TRANSFORMING GROWTH FACTOR (TGF)-β SIGNALING INDUCES LEFT VENTRICULAR DILATION AND DYSFUNCTION IN THE PRESSURE OVERLOADED HEART

1. Effects of 7, 28, and 120 days of TAC and/or 25 mM ZnSO₄ in drinking water on left (LV) and right (RV) ventricular weights and LV cardiomyocyte (CM) size of DnTGFβRII and NTG mice. .......................... 48
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
</table>

## Introduction

1. TGF-β/ANP/AngII Signaling Pathway ............................................................... 6

2. Model of Transverse Aortic Constriction (TAC) ............................................. 13

### INHIBITION OF TRANSFORMING GROWTH FACTOR (TGF)-β SIGNALING INDUCES LEFT VENTRICULAR DILATION AND DYSFUNCTION IN THE PRESSURE OVERLOADED HEART

1. Representative Light micrographs of the left ventricle (LV) ......................... 42

2. Effects of 7 days of TAC or sham operation (Control) on density of Ki67 positive interstitial non-myocytes ................................................................. 43

3. Representative picrosirius red stained cross sections at a level below the mitral valve of the posterior wall ................................................................. 44

4. Effects of 28 and 120 days of TAC on interstitial collagen volume in LV ........ 45

5. Representative micrographs of 2D echocardiography of LV .......................... 46

6. Effects of 120 days of TAC on (A) body weight BW, (B) LV/BW ratio, (C) left ventricular end diastolic dimension (LVEDD), (D) left ventricular end systolic dimension (LVESD), (E) end diastolic volume (EDV), (F) end systolic volume (ESV), (G) ejection fraction (EF), (H) fractional shortening (FS) ........................................................................................................... 47
ANP SIGNALING INHIBITS TGF-B INDUCED SMAD2 AND SMAD3 NUCLEAR TRANSLOCATION AND EXTRACELLULAR MATRIX EXPRESSION IN RAT PULMONARY ARTERIAL SMOOTH MUSCLE CELLS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>75</td>
</tr>
<tr>
<td>2</td>
<td>77</td>
</tr>
<tr>
<td>3</td>
<td>78</td>
</tr>
<tr>
<td>4</td>
<td>79</td>
</tr>
<tr>
<td>5</td>
<td>80</td>
</tr>
<tr>
<td>6</td>
<td>81</td>
</tr>
</tbody>
</table>

1. (A & B) Dose-dependent stimulatory effects of TGF-β1 on steady-state periostin (PN) and osteopontin (OPN) mRNA expression in rat PASMCs (C, D & E) Inhibitory effects of ANP or cGMP on TGF-β1-stimulated PN, OPN and PAI-1 mRNA expression in rat PASMCs. E) subgroups of PASMCs were pretreated with PKG inhibitors. F) Cell lysates (25 μg) were size fractionated by SDS-PAGE, and Western blot analysis was performed with selective anti-PN, OPN, and β-actin antibodies.

2. Representative Western blot analysis demonstrating that A) pretreatment with cGMP does not block TGF-β1-induced phosphorylation of Smad2 or Smad3 and B) Neither cGMP nor TGFβ1 treatment altered total Smad2/3 levels in rat PASMCs.

3. Representative fluorescent micrographs show that ANP and cGMP inhibit TGF-β1-stimulated nuclear translocation of A) pSmad2 and B) pSmad3 in rat PASMCs.

4. Confocal fluorescent micrographs show that cGMP inhibits TGF-β1-stimulated nuclear translocation of A) pSmad2 and B) pSmad3 in rat PASMCs.

5. (A & B) Inhibitory effects of ANP and cGMP on TGF-β1-stimulated nuclear translocation of pSmad2 and pSAMD3 in rat PASMCs. (C & D) Time course of the inhibitory effects of cGMP on TGF-β1-stimulated nuclear translocation of pSmad2 and pSmad3 in rat PASMCs.

6. Effects of ANP, cGMP, TGF-β1, and/or PKG inhibitors on PKG activity in rat PASMCs.
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>PKG inhibitors KT5823 and Rp-8-Br-cGMP block the inhibitory effects of ANP and cGMP on TGF-β1 (1 ng/ml)-stimulated nuclear translocation of pSmad3 in rat PASMCs ................................................................. 82</td>
</tr>
<tr>
<td>8</td>
<td>Schematic illustration of TGF-β1 signal transduction and postulated mechanisms of inhibition by ANP-cGMP-PKG signaling ................................. 83</td>
</tr>
</tbody>
</table>

**ANP INHIBITS TGF-β-INDUCED SMAD SIGNALING AND MYOFIBROBLAST TRANSFORMATION IN MOUSE CARDIAC FIBROBLASTS**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Representative micrographs of collagen and α-smooth muscle actin positive myofibroblasts in LV of male Nppa^-/- and Nppa^+/+ mice one week post TAC ................................................................. 110</td>
</tr>
<tr>
<td>2</td>
<td>Representative micrographs (400X) and (E) bar graphs of means±SE of α-SMA-stained cultured mouse CFs treated with TGF-β1 and/or cGMP ................................................................. 111</td>
</tr>
<tr>
<td>3</td>
<td>(A) Effects of ANP (0.1 µM for 30 min) on intracellular cGMP levels in mouse and rat CFs. (B) to (D) Effects of ANP (1 µM) or cGMP (1 mM) on TGF-β1 (5 ng/ml) stimulated collagen synthesis, cell proliferation and PAI-1 mRNA expression in mouse CFs .................................... 112</td>
</tr>
<tr>
<td>4</td>
<td>(A-D) Representative micrographs show that cGMP inhibits TGF-β1-stimulated nuclear translocation of pSmad3 in mouse CFs ................................................................. 113</td>
</tr>
<tr>
<td>5</td>
<td>(A) Representative Western blots and averaged bar graphs of pSmad3 demonstrating that cGMP does not block TGF-β1-induced phosphorylation of Smad3 and Smad2 in mouse CFs. (B) Radioautographs and Western blots of purified Smad3 protein that was phosphorylated with PKG (20 min) and then digested with carboxypeptidase Y for 1, 30, and 60 min ............................................. 114</td>
</tr>
<tr>
<td>6</td>
<td>MS/MS analysis of Smad3 that has been phosphorylated with PKG ........ 115</td>
</tr>
</tbody>
</table>
LIST OF FIGURES (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>DISCUSSION AND FUTURE DIRECTIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Representative Western blots and averaged bar graphs for MAPK proteins on Ang II induced Smad3 activation in mice cardiac fibroblasts ................................................................. 123</td>
</tr>
<tr>
<td>2</td>
<td>Representative Western blots and averaged bar graphs for MAPK proteins on Ang II induced Smad3 activation in rat pulmonary artery smooth muscle cells ......................................................... 124</td>
</tr>
<tr>
<td>3</td>
<td>Schematic illustration of TGFβ/Smad and Ang II signaling ....................................... 125</td>
</tr>
</tbody>
</table>
**GENERAL LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>analysis of covariance</td>
</tr>
<tr>
<td>ANP</td>
<td>Atrial Naturetic Peptide</td>
</tr>
<tr>
<td>BNP</td>
<td>B-type naturetic peptide</td>
</tr>
<tr>
<td>CF</td>
<td>cardiac Fibroblast</td>
</tr>
<tr>
<td>CO</td>
<td>cardiac output</td>
</tr>
<tr>
<td>DnTGFβRII</td>
<td>Dominant negative transforming growth factor beta type II receptor</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDV</td>
<td>end diastolic volume</td>
</tr>
<tr>
<td>EF</td>
<td>ejection fraction</td>
</tr>
<tr>
<td>ESV</td>
<td>end systolic volume</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FS</td>
<td>fractional shortening</td>
</tr>
<tr>
<td>FT-ICR MS</td>
<td>fourier transform cyclotron resonance mass spectrometry</td>
</tr>
<tr>
<td>H2O</td>
<td>water</td>
</tr>
<tr>
<td>HR</td>
<td>heart rate</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
<tr>
<td>LAP</td>
<td>Latent associated peptide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>LTBP</td>
<td>Latent TGFβ binding protein</td>
</tr>
<tr>
<td>LV</td>
<td>left ventricle</td>
</tr>
<tr>
<td>LVEDD</td>
<td>left ventricular end diastolic dimension</td>
</tr>
<tr>
<td>LVESD</td>
<td>left ventricular end systolic dimension</td>
</tr>
<tr>
<td>LVH</td>
<td>left ventricular hypertrophy</td>
</tr>
<tr>
<td>MF</td>
<td>myofibroblast</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MS/MS</td>
<td>ion trap tandem mass spectrometry</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NP</td>
<td>natriuretic peptide</td>
</tr>
<tr>
<td>NTG</td>
<td>non-transgenic</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen Activator Inhibitor</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pSmad</td>
<td>phosphorylated SMAD</td>
</tr>
<tr>
<td>PASMCs</td>
<td>pulmonary artery smooth muscle cell</td>
</tr>
<tr>
<td>OPN</td>
<td>osteopontin</td>
</tr>
<tr>
<td>PN</td>
<td>periostin</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>RV</td>
<td>right ventricle</td>
</tr>
<tr>
<td>SMAD</td>
<td>Small Mothers Against Dicapentalgia</td>
</tr>
<tr>
<td>TAC</td>
<td>Transverse aortic constriction</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitors of MMPs</td>
</tr>
<tr>
<td>TSP-1</td>
<td>Thrombospondin-1</td>
</tr>
<tr>
<td>VSMCs</td>
<td>vascular smooth muscle cell</td>
</tr>
<tr>
<td>Zn^{++}</td>
<td>zinc sulfate</td>
</tr>
</tbody>
</table>
INTRODUCTION

Pressure Overload-Induced Cardiac Remodeling and Fibrosis

Hypertensive heart disease, including heart failure, is a leading cause of morbidity and mortality in industrialized societies (15). It is increasing in prevalence due to longer life expectancy and partially successful antihypertensive treatment, which prevents fatal events but does not fully control blood pressure. Aortic stenosis, a classic cause of pressure induced pathology in the heart, can also lead to heart failure by causing pressure overload-induced left ventricular (LV) hypertrophy, remodeling and fibrosis. Existing pharmacologic therapies for pressure overload-induced heart diseases are costly, limited in efficacy, and burdened by unfavorable adverse effect profiles. In particular, there is no successful treatment for heart failure with preserved LV function (diastolic dysfunction), now a common occurrence in elderly persons with longstanding hypertension (30). Accordingly, additional investigation into the basic pathophysiology and mechanisms of pressure overload-induced cardiac hypertrophy, fibrosis and remodeling is needed in order to develop the rationale for novel therapeutic strategies.

In the pulmonary vascular system, the responses to stress bear some similarities to those of the heart. When patients are exposed to decreased oxygen levels, the pulmonary arteries supplying areas of the lung where the
oxygen supply is most limited vasoconstrict, thus shunting blood to areas of the lung that can oxygenate the blood properly. This vasoconstrictor response increases the pressure in the pulmonary artery and right ventricle, a syndrome referred to as hypoxia induced pulmonary hypertension. The right ventricle must then contract more forcibly in order to pump the blood through the lungs, resulting in right ventricular hypertrophy and remodeling, which if left untreated can result in right heart failure. The increased pulmonary artery pressure is sustained by remodeling of resistance vessels in lung, characterized by increased extracellular matrix (ECM) formation, fibrotic thickening of the arterial wall and neomuscularization of small arteries. We have used a murine model of chronic normobaric hypoxic exposure and isolated pulmonary arterial smooth muscle cells exposed to hypoxic atmospheres in culture to examine the mechanisms of these responses to hypoxia.

Both the characteristic pressure overload-induced cardiac hypertrophy/remodeling and the hypoxia-induced pulmonary arterial hypertrophy/remodeling lesions have been attributed to perturbations in the normal relationships between pro-fibrogenic/mitogenic and anti-fibrogenic/growth-inhibiting factors (2, 10, 33, 34). Hemodynamic and hypoxic stress induces cardiac and pulmonary arterial remodeling, at least in part, by activating many cytokines and growth factors. The overall hypothesis for this report is that during pressure overload and hypoxia stress, expression of both atrial natriuretic peptide (ANP) and transforming growth factor beta (TGF-β) are enhanced in wild type mice; the pro-fibrogenic factor, TGF-β1, contributes to
cardiac and pulmonary vascular remodeling with increased ECM formation and fibrosis, while the increased ANP plays a counterregulatory role and protects against these events.

**Transforming Growth Factor (TGF)-β**

Transforming growth factor (TGF)-β (a 30+ member superfamily) is a profibrogenic factor and mediator of tissue fibrosis, repair, cellular differentiation, transformation, growth and migration, and plays an important role in cardiac and pulmonary remodeling under stress conditions (37, 38, 58). All TGF-β isoforms signal through the membrane bound heterotrimeric TGF-β receptors type I and II (TGFβRI and TGFβRII) in virtually all mammalian cells (Figure 1). TGF-β1, the major isoform in heart (1, 55, 58), is expressed in cardiac fibroblasts (CFs) and cardiac myocytes (CMs) (1) and stimulates CF transformation (to myofibroblasts [MFs]) and proliferation, as well as ECM production (6, 47). TGF-β1, also the major isoform in lung, has profound effects during adaptation to hypoxic stress in lung. Under hypoxic stress conditions, TGFβ promotes ECM deposition in the pulmonary arterial tree, resulting in pulmonary vascular remodeling and sustained pulmonary hypertension.

TGF-β1 is synthesized as a 391-amino acid precursor molecule which includes two 112 amino acid subunits that become mature TGF-β, and a latent associated peptide (LAP). This complex binds to the latent TGF-β binding protein (LTBP) and is secreted out of the cell into the ECM, where it remains in an inactive state. Once the LTBP and the LAP are cleaved, the TGF-β protein is
activated, binds to its receptors, and exerts its biological effects (40, 41, 52). Molecules that have been identified as TGF-β activators include matrix metalloproteinases (MMP)-2 and MMP-9 and thrombospondin 1 (TSP-1). When CMs are stretched due to pressure overload stress, they secrete TSP-1, which activates the latent TGF-β1. Active TGF-β1 binds membrane receptors that activate downstream signaling molecules Smad2 and Smad3, which are phosphorylated on the C-terminal serine residues (Figure 1). Phosphorylated Smad2 and Smad3 (pSmad2 and pSmad3) bind to Smad4 and translocate to the nucleus. The Smad complex then binds to response elements in the promoter regions of the ECM genes and activates pro-fibrogenic factors by upregulating gene transcription.

Mice overexpressing TGF-β1 have cardiac hypertrophy and interstitial fibrosis (49), and TGF-β1 expression is increased in hearts subjected to pressure overload (55, 56, 58). Under hemodynamic stress, CFs undergo MF transformation, proliferate and express ECM proteins. TGF-β, which is activated in the stressed myocardium, plays a role in these processes (39).

TGFβ1 is also an important player in hypoxia induced pulmonary vascular remodeling and fibrosis. It has been shown, using a dominant negative transforming growth factor (TGF)-β transgenic mouse model that expresses an inducible mutation of the TGF-β type II receptor, the stimulatory effects of hypoxic exposure on pulmonary arterial smooth muscle cell proliferation and lung ECM protein expression were abrogated in DnTGFβRII mice given ZnSO₄ (to induce the mutation) in comparison to wild type controls. This study supports
the conclusion that TGF-beta signaling plays an important role in hypoxia-induced pulmonary vascular adaptation in the newborn animal model (2).

**Atrial Natriuretic Peptide**

ANP, a peptide synthesized and stored in granules in the atrial myocyte, is released in response to a stimulus (e.g., stretch) and binds to guanylate cyclase-coupled membrane natriuretic peptide (NP) receptors, which causes increases in intracellular cGMP levels and activates cGMP–dependent protein kinase G (PKG), with resultant growth-inhibiting and antiproliferative effects in a variety of cell types, including PASMCs and CFs (10). ANP is a well-known component of the fetal gene program that is overexpressed in adult heart under stress conditions. ANP and the NP receptors are expressed in CFs (8, 28), and ANP can stimulate cGMP expression in mouse CFs in vitro, indicating a direct paracrine/autocrine effect. ANP is not produced in the lung, but shuttles to the lung from the cardiac atria when exposed to stress. ANP promotes natriuresis and diuresis, and inhibits growth, proliferation, and ECM protein expression and induces apoptosis in a variety of cell types, including CFs, CMs, and PASMCs.
Figure 1. Schematic illustration of the cardioprotective (inhibitory) role(s) of ANP in modulating pressure overload-induced cardiac remodeling and fibrosis, ultimately leading to failure. We hypothesize that under pressure overload stress, increased TGF-β initiates, and ANP retards, CF transformation to active myofibroblasts. In activated CF, ANP acts as an anti-fibrogenic factor, and TGF-β, ANGII, and FGF act as pro-fibrogenic factors, to regulate ECM (e.g. collagens, PN, OPN, fibronectin, and thrombospondin) production and turnover. Without the counter regulatory/inhibitory effects of the ANP-cGMP-PKG pathway, Nppa-/- and Npr1-/- mice with TAC develop exaggerated CF proliferation/transformation, increased ECM deposition/turnover, more severe cardiac remodeling/fibrosis, and eventually heart failure in response to pressure overload. Abbreviations: NPRA- natriuretic peptide receptor A, TβRI and TβRII- TGFβ-R1 and TGFβ-RII receptors, GPCR-G protein coupled receptor, RTK-receptor tyrosine kinase, PKG- protein kinase G, MKK-MAPK kinase, ERK-extracellular signal-regulated kinase, JNK- c-Jun N-terminal kinase, PI3K-phosphatidylinositol 3 kinase, Akt- protein kinase B, S6K- ribosomal S6 kinase, CTGF- connective tissue growth factor, MMP-matrix metalloproteinase, TIMP-tissue inhibitor of MMP, PAI-1-plasminogen activator-inhibitor.
Expression of ANP in heart is inversely related to cardiac growth/remodeling (14, 19, 21, 25, 27, 54). Transgenic mice overexpressing ANP have smaller hearts than wild type mice (26). Further, previous observations from our laboratory demonstrate that ANP is upregulated in heart under pressure or volume overload stress and is functionally active in modulating stress-induced increases in fibrosis and remodeling. Mice with homozygous deletion of the pro-ANP gene (Nppa\(^{-/-}\)) exhibit cardiac fibrosis under resting conditions (19, 20, 43, 59) and develop exaggerated fibrosis and LV remodeling after pressure or volume overload compared to wild type control mice (20, 43, 59). These abnormalities appear in attenuated form in heterozygous Nppa\(^{+/-}\) mice subjected to pressure overload stress, indicating that even partial ANP deficiency results in adverse cardiac remodeling under hemodynamic stress (20). In contrast to ANP, B-type natriuretic peptide (BNP) appears not to modulate cardiac hypertrophy. Circulating BNP is not increased, and BNP is unable to compensate for the lack of ANP in Nppa\(^{-/-}\) mice (57). BNP\(^{-/-}\) mice do not develop cardiac hypertrophy or systemic hypertension (44, 58). These findings suggested that ANP deficiency alone is sufficient to generate cardiac hypertrophy, particularly under conditions of hemodynamic stress.

We previously used the DnTGF\(\beta\)RII mouse model to determine the importance of TGF-\(\beta\) signaling in chronic hypoxia induced pulmonary hypertension (2, 11). The most striking finding of the study was that disruption of TGF-\(\beta\) Type II receptor expression in lung greatly attenuated the chronic hypoxia-induced phenotypic changes (pulmonary hypertension, RV enlargement,
pulmonary arterial remodeling and muscularization, myofibroblast transformation of alveolar fibroblasts, and ECM mRNA and protein expression) in DnTGFβRII mice compared to NTG controls. These dramatic findings support our hypothesis that TGF-β signaling plays an important role in the pulmonary response to chronic hypoxic stress (11).

We hypothesize that the profibrogenic phenotype that results from eliminating the anti-fibrotic ANP/cGMP/PKG signaling pathway is related to unopposed TGF-β signaling in response to pressure overload or hypoxic stress (Figure 1). Our laboratory has shown that ANP and TGF-β play important counterregulatory roles in pressure overload-induced cardiac remodeling and fibrosis (20, 42, 43) and in hypoxia induced pulmonary hypertension, remodeling, and fibrosis (10).

**CARDIAC FIBROBLASTS AND THE ECM**

CFs, the most abundant cells in heart (39), affect myocardial function by synthesizing and depositing ECM proteins, thus anchoring the CMs and influencing both systolic and diastolic function. CFs are the cell type primarily responsible for homeostatic maintenance of the ECM, and therefore the compliance of the myocardium. We have shown remarkable increases in numbers of transformed cardiac fibroblasts (CFs) and expression of ECM molecules (collagen I and III, fibronectin, osteopontin [OPN], thrombospondin and peristin [PN]) in hearts of Nppa−/− mice subjected to TAC compared to wild type mice subjected to a similar stress (59).
The ECM is maintained, in part, by ongoing cycles of synthesis and degradation of collagen, one of the main components of the ECM. Five isoforms of collagen are present in the myocardium: type I, III, IV, V, and VI (37). Collagen I represents approximately 80% of all collagen synthesized by the CFs.

Collagen biosynthesis is regulated transcriptionally by fibrogenic growth factors such as TGF-β, and degradation of collagen is carried out by matrix metalloproteinases (MMPs). TGF-β increases the abundance of mRNA for collagen types I and III in the whole heart and enhances collagen type I synthesis in CFs in culture (16, 17). The dynamic plasticity of the ECM is maintained not only by changes in collagen structure and accumulation, but also by MMPs that degrade collagen and other matrix components. In cardiac tissue, collagenases degrade intact fibrillar collagen, rendering fibrils unable to maintain stable formation and promoting further degradation by MMPs (32, 35, 36). Under pressure overload stress, synthesis and degradation of collagen are altered, leading to fibrosis and eventual cardiac dysfunction.

Tissue inhibitors of matrix metalloproteinases (TIMPs) counterbalance MMP-activation pathways by binding to the active site of the MMPs, thereby blocking their binding to the ECM substrate (35, 36). Altered expression and activity of various MMPs and TIMPs have been detected during cardiac remodeling induced by a variety of stimuli (31, 53). Expression of MMPs and TIMPs is correlated with the development of cardiac hypertrophy and failure in rodent heart (31, 53).
The production of ECM proteins such as collagen, periostin and osteopontin, and MMPs and TIMPs, and their regulators determines the progression of the fibrotic process in heart. In the injured heart, the CF transforms into the MF phenotype, the activated form of the CF. The MF expresses ECM proteins such as periostin and osteopontin, as well as alpha-smooth muscle actin, and its phenotype is strongly promoted by the pro-fibrogenic factor TGF-β, resulting in cardiac remodeling and fibrosis. It has been shown that the administration of function-blocking antibodies of TGF-β1 prevents MF conversion and subsequent increases in mRNA encoding collagen I in pressure overloaded rat hearts (16, 17).

PULMONARY ARTERY SMOOTH MUSCLE CELLS AND THE ECM

TGF-β is a key mediator of pulmonary morphogenesis and of the pathogenesis of pulmonary fibrosis and vascular remodeling (3, 18, 60). The inactive form of TGF-β is present in the lung in small amounts, and levels of active TGF-β increase under pathological stimuli, such as hypoxia, to promote pulmonary hypertension and pulmonary vascular remodeling. The atrial myocytes release ANP to counteract the increasing atrial distention due to the retrograde increase in pulmonary pressure as a counterregulator of rising TGF-β activity. The functional significance of the hypoxia-induced increase in ANP is supported by observations of worsening pulmonary hypertension and vascular remodeling in Nppa−/− mice compared with wild type controls in response to hypoxic stress (54). We have previously shown that infusing ANP into Nppa−/−
mice prevents the extreme pulmonary arterial remodeling exhibited in the ANP deficient mice in comparison to wild type controls (23, 24, 54).

Further, using the DnTGFβRII transgenic mouse model, blocking endogenous TGF-β signaling in the lung greatly attenuates chronic hypoxia-induced phenotypic changes (i.e. pulmonary hypertension, right ventricular enlargement, and pulmonary vascular remodeling) (2, 11). In order to test the current hypothesis that TGF-β promotes ECM expression in isolated PASMCs and ANP exerts a counterregulatory role to protect against these events, we used TGF-β to stimulate PASMCs, and investigated the counterregulatory role of ANP on ECM production, nuclear translocation and phosphorylation of the Smad proteins.

ANIMAL MODELS

The transgenic mouse models used in the in vivo sections of this report were the: 1) The dominant negative transforming growth factor beta type II receptor (DnTGFβRII) mouse and 2) the atrial natriuretic peptide deficient (Nppa⁻/⁻) mouse. The DnTGFβRII mouse utilizes an inducible mutation of the type II receptor that inhibits the endogenous TGF-β/Smad signaling pathway (11). The mutated gene has a 4X heavy metal promoter that is induced by intake of ZnSO₄ in the drinking water. The mutated gene has the extracellular domain of the type II receptor but lacks the intracellular domain for downstream signaling. Therefore, the TGF-β ligand is competitively inhibited, reducing the downstream signaling. By attenuation of this pathway there is a reduction of cardiac
remodeling and fibrosis and pulmonary vascular remodeling under stress conditions.

We have shown that disruption of TGF-β signaling greatly attenuates pressure overload-induced MF transformation and interstitial fibrosis in heart, supporting a critical role for TGF-β signaling in the pathogenesis of pressure overload-induced cardiac hypertrophy and remodeling (34). This current study shows that inhibition of TGF-β signaling results in attenuation of collagen deposition and nonmyocyte cell proliferation during pressure overload-induced stress, with eventual development of cardiac dysfunction at 120 days of pressure overload.

Our previous studies have shown that ANP and TGF-β are upregulated in heart and lung of rats exposed to chronic normobaric hypoxia (54). The significance of the hypoxia induced increase in ANP is supported by the observation of worsening pulmonary hypertension and vascular remodeling in Nppa−/− mice compared with wild type controls in response to chronic hypoxia (54). In this in vitro report in isolated rat PASMCs, we found that ANP/cGMP/PKG signaling directly inhibits TGF-β/Smad signaling by blocking nuclear translocation of pSmad3 (33). We also used the Nppa−/− mouse in this report to focus on MF transformation and collagen deposition, both of which are increased in hearts of Nppa−/− mice compared with Nppa+/+ mice subjected to pressure overload.
The experimental model used in this in vivo study was transverse aortic constriction (TAC) induced pressure overload in the mouse. (Figure 2) Using a sternal surgical approach, the thoracic aorta is exposed and a 0.4 mm wire is placed on the aorta. Using a 7.0 suture, a surgeons knot is tied around the wire and the arch of the aorta, the wire is then removed to make the same size constriction consistently (59). Pressure gradients across the stricture have been measured at 50-65 mmHg using this method (59).

In this report we used the transgenic models of the DnTGFβII and Nppa/− mice for in vivo studies of MF transformation and collagen deposition, and rat PASMCs and mouse CFs for in vitro study of nuclear translocation, Smad phosphorylation and ECM expression. We have shown that ANP and TGF-β play important counterregulatory roles in pressure overload and hypoxia-induced hypertrophy and fibrosis in heart, and in pulmonary vascular remodeling.

Figure 2. Model of Transverse Aortic Constriction (TAC). Pressure gradients of 50-60 mmHg across stricture. (59).
PROJECT SUMMARY

Our general hypothesis is that during stress, expression of both ANP and TGF-β are enhanced in wild type mice; the pro-fibrogenic/growth factor TGF-β contributes to cardiac remodeling with increased interstitial and perivascular fibrosis and eventual cardiac dysfunction, while the anti-fibrogenic/growth factor ANP plays a counterregulatory role and protects against these events. We have found that TGF-β promotes CF differentiation and proliferation and ECM expression under stressful conditions (34), and our current data suggest that interstitial collagen resulting from enhanced TGF-β signaling may be required to maintain LV structure and function under chronic pressure overload. The rationale for this project is that creating a better understanding of the pathogenesis of cardiac fibrosis in the environment of pressure overload-induced cardiac stress is needed in order to develop novel preventive and therapeutic strategies for this serious disease process.

Similar to the cardiac response to pressure overload, the lung exhibits upregulation of TGF-β when exposed to chronic hypoxia, resulting in pulmonary hypertension and pulmonary vascular remodeling. We have observed a direct interaction between the ANP/cGMP/PKG (anti-fibrotic) and TGF-β (pro-fibrotic) pathways in PASMCs (33) in adapting to hypoxic stress. TGF-β accelerates ECM production in hypoxia exposed PASMCs, and activation of ANP signaling via cGMP or ANP administration inhibits these processes by interrupting TGF-β1 signaling at the level of Smad3 by overphosphorylation, resulting in blockade of
nuclear translocation of pSmad. Elucidating the functional interactions between TGF-β and ANP signaling cascades in the setting of chronic hypoxia in lung is important in achieving a better understanding of the molecular pathogenesis of pulmonary hypertension, RV hypertrophy and pulmonary vascular remodeling. The rationale for this project is to achieve a better understanding of the pathogenesis of pulmonary vascular remodeling in order to develop preventive and therapeutic interventions for pulmonary hypertension in humans, a serious, debilitating and relatively untreatable disease.
Inhibition of Transforming Growth Factor (TGF)-β Signaling Induces Left Ventricular Dilation and Dysfunction in the Pressure Overloaded Heart

by

Jason A. Lucas, Yun Zhang, Peng Li, Kaizheng Gong, Andrew P. Miller, Erum Hassan, Fadi Hage, Dongqi Xing, Bryan Wells, Suzanne Oparil, Yiu-Fai Chen

Submitted to American Journal of Physiology- Heart and Circulation

Format adapted for dissertation
ABSTRACT

This study utilized a transgenic mouse model that expresses an inducible dominant-negative mutation of the transforming growth factor (TGF)-β type II receptor (DnTGFβRII) to define the structural and functional responses of the left ventricle (LV) to pressure overload stress in the absence of an intact TGF-β signalling cascade. DnTGFβRII and nontransgenic (NTG) control mice (male, 8-10 wk) were randomized to receive Zn++ (25mM ZnSO₄ in drinking H₂O to induce DnTGFβRII gene expression) or control tap H₂O, then further randomized to undergo transverse aortic constriction (TAC) or sham surgery. At 7 days post TAC, interstitial non-myocyte proliferation, (Ki67 staining) was greatly reduced in LV of DnTGFβRII+Zn++ mice compared to the other TAC groups. At 28 and 120 days post TAC, collagen deposition (picrosirius-red staining) in LV was attenuated in DnTGFβRII+Zn++ mice compared to the other TAC groups. LV end systolic diameter (LVESD) and end systolic and end diastolic volumes (EDV and ESV) were markedly increased, while ejection fraction (EF) and fractional shortening (FS) were significantly decreased in TAC-DnTGFβRII+Zn++ mice compared to the other groups at 120 days post TAC. These data indicate that interruption of TGF-β signaling attenuates pressure overload-induced interstitial non-myocyte proliferation and collagen deposition and promotes LV dilation and dysfunction in the pressure overloadeed heart, and thus creating a novel model of dilated cardiomyopathy.

Key Words: Transforming growth factor beta, Cardiac hypertrophy, Smad protein
INTRODUCTION

Pressure-overload stress (e.g. hypertension or aortic stenosis) results in excessive cardiac fibrosis, changes in left ventricular (LV) geometry, and disruption in LV contractility, ultimately leading to heart failure (16,3). Our previous studies have shown that transforming growth factor (TGF)-β plays an important role in pressure overload–induced cardiac hypertrophy, remodeling, and fibrosis (20). TGF-β expression is upregulated in LV of mice subjected to pressure-overload stress induced by transverse aortic constriction (TAC) (36). The pressure overload-induced increase in TGF-β is accompanied by LV hypertrophy, fibrosis, and remodeling that is exaggerated in the absence of anti-fibrotic and growth inhibiting effects mediated by the atrial natriuretic peptide (ANP) signaling pathway. Thus, the ANP null mouse (Nppa-/-) exhibits a profibrogenic phenotype that appears to be related to unopposed TGF-β signaling (36,20,12).

TGF-β1, the major isoform in the heart (35,6), stimulates cardiac fibroblast transformation (to myofibroblasts) and proliferation, as well as extracellular matrix (ECM) production (22,28). Mice over expressing TGF-β1 have cardiac hypertrophy and interstitial fibrosis (29), and TGF-β1 expression is increased in hearts subjected to pressure overload (35,33,6,20). TGF-β signals through the membrane bound heterotrimeric TGF-β receptors type I and II (TGFβRI and TGFβRII). When the TGFβRI and RII are activated, downstream signaling molecules Smad2 and Smad3 are phosphorylated on the C-terminal serine residues. Phosphorylated Smad2 and Smad3 (pSmad2 and Smad3) bind to
Smad4 and translocate to the nucleus (32). The Smad complex then binds to response elements in the promoter regions of the ECM genes and activates profibrogenic factors by upregulating gene transcription.

Our present study utilized a novel mouse model that expresses an inducible dominant negative mutation of the TGF-β receptor type II gene (DnTGFβRII), and thus cannot activate the TGF-β/Smad signaling cascade (31), to characterize the effect of blocking TGF-β signaling on the phenotype of the pressure overloaded LV. We observed that induction of DnTGFβRII expression attenuates pressure overload-induced interstitial non-myocyte proliferation and collagen deposition and promotes LV dilation and dysfunction in the mouse, resulting in a novel model of dilated cardiomyopathy.
METHODS

Transgenic Mice and Animal Preparation

Male DnTGFβRII transgenic mice originally generated by Dr. Rosa Serra (31) and nontransgenic (NTG) C57/BL6 mice were studied. All mice were raised in our resident colony, which was founded with pathogen-free breeding pairs. Genotypes were identified by polymerase chain reaction (PCR) assay of genomic DNA from tail snips after weaning (2). The DnTGFβRII mouse expresses a cytoplasmically truncated TGFβRII receptor that competes with endogenous receptors for heterodimeric complex (TGFβRI and –RII) formation and is thus a dominant-negative mutant (31). The DnTGFβRII lacks the cytoplasmic kinase domain and has no intrinsic activity. Over expression of DnTGFβRII is under the control of a metallothionein-derived promoter that can be induced by Zn++.

Expression of DnTGFβRII was induced by giving 25 mM ZnSO₄ in the drinking water to DnTGFβRII mice (DnTGFβRII+Zn++) beginning 1 wk prior to transverse aortic constriction (TAC) or sham surgery (36) and continuing throughout the study. NTG mice drinking either ZnSO₄ water (NTG+Zn++) or double distilled H₂O (NTG+H₂O) served as controls. DnTGFβRII mice drinking double distilled H₂O (DnTGFβRII+H₂O) with TAC or sham surgery were used as additional controls. These 8 groups of mice (Sham-NTG+H₂O, TAC-NTG+H₂O, Sham-NTG+Zn++, TAC-NTG+Zn++, Sham-DnTGFβRII+H₂O, TAC-DnTGFβRII+H₂O, Sham-DnTGFβRII+Zn++, TAC-DnTGFβRII+Zn++) were fed a standard diet (Harlan-Teklad) and were housed in rooms maintained at a
constant humidity (60±5%), temperature (24±1ºC), and light cycle (6:00 AM-6:00 PM).

Induction of DnTGFβRII expression was confirmed by detecting the DnTGFβRII receptor mRNA in heart, lung and liver of DnTGFβRII mice given ZnSO₄ (or H₂O as a negative control) using RT-PCR (primers: 5’-ATC GTC ATC GTC TTT GTA GTC-3’ and 5’-TCC CA C CGC ACG TTC AGA AG-3’) as described previously (2). All protocols were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham and were consistent with the Public Health Service Policy on Humane Care and Use of Laboratory Animals (Office of Laboratory Animal Welfare, August 2002) and the Guide for the Care and Use of Laboratory Animals published by National Institutes of Health (NIH Publication No. 96-01, revised in 2002).

**Surgical Procedures**

Male DnTGFβRII and NTG mice, 8-10 wks of age, were anesthetized with an intraperitoneally administered mixture of ketamine (80 mg/kg) and xylazine (12 mg/kg), and TAC was performed as described previously (36). The aortic band was located between the proximal left carotid artery and the brachiocephalic arteries on the ascending aorta. Pressure gradients across the TAC were 50-60 mmHg, and similar among genotypes, as described previously (36). Sham-operated mice served as controls.

**Tissue Collection**

Separate groups of mice were sacrificed at 7, 28 and 120 days after TAC with an overdose of ketamine/xylazine followed by cervical dislocation. Hearts,
lungs, liver, and kidneys were quickly removed and the LV and right ventricle (RV) were dissected carefully and weighed. LV sections were divided into two portions: the apical portion was fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned for histological analysis; the basal portion was immediately frozen in liquid N₂ and stored at -80°C for biochemical analysis.

**Histological Analysis**

At 7 days after TAC, cell proliferation and apoptosis were assessed in paraffin-embedded LV cross sections using Ki-67 and TUNEL reagent (Vector Lab), respectively, according to the manufactures’ instruction. Proliferative or apoptotic indices were determined by counting the number of Ki67- or TUNEL-stained nuclei in 400X microscopic fields of posterior wall and septum of each LV. The identity of the samples was masked to the two examiners to avoid bias. Twelve randomly selected high-power fields from each mouse were examined and quantitated.

LV cardiomyocyte area was measured in 28 day samples of LV as previously described (26). Morphometric analysis of each heart section was performed with a computer-based morphometric system (Motic Image Plus 2.0). Four hearts from each experimental group were included in the histological analysis. At least five hematoxylin and eosin stained cross sections of each heart were examined. 80 cardiomyocytes from each LV were measured in nucleated transverse sections and the average area of the 80 myocytes was calculated. All morphometric analyses were carried out by a single examiner,
who was blinded with respect to the experimental group to which each sample belonged.

**Collagen Volume**

At 28 and 120 days after TAC, LV interstitial collagen volume percentage, at the level below the mitral valve, was measured in picrosirius red (0.1%)-stained cross sections as described previously (36,13,12), using a microscopic system with a green (540 nm) filter to enhance contrast for computer imaging analysis. Quantitative morphometric analysis of collagen content was carried out by light microscopy with a Qimaging QiCam digital camera (Qimaging) interfaced with a computer system running Metamorph 6.2v4 software (Universal Imaging). At least 12 randomly selected images (400X) from the posterior wall and septum of each LV were analyzed. The identity of the samples was masked to the two examiners to avoid bias.

**Echocardiography**

At 120 days after TAC or sham surgery, echocardiography was performed on isofluorane anesthetized mice with a 15-MHz transducer (Philips) and a commercially available ultrasound system (Phillips Sonos 5500) as described previously (11,26,13,12). LV end-diastolic dimension (LVEDD), LV end-systolic dimension (LVESD), and septal (intraventricular septal [IVS]) and posterior wall (PW) diastolic thickness were measured by 2D-guided M-mode echocardiography from the parasternal long-axis view. LV end-diastolic volume (EDV), end-systolic volume (ESV), ejection fraction (EF), fractional shortening (FS), and cardiac output (CO) were calculated as EDV=
7xLVEDD³/(2.4+LVEDV), ESV = 7 x LVESD³/(2.4+LVESV), FS = (LVEDD-LVESD)/LVEDDx100, EF = (EDV-ESV)/EDVx100, CO = heart rate (HR)x(EDV-ESV)/1000, respectively (11). A single examiner, blinded to genotype and treatment, interpreted all studies.

Statistical Analysis

Results were expressed as mean±SEM. Analyses were carried out using the SigmaStat statistical package. Tissue weight and echocardiographic measurements were normalized by ANCOVA with body weight as the covariate (Packard 1988). Our primary statistical test was ANOVA, 1-way ANOVA to evaluate the differences in mean values due to main effects (genotype, TAC, or Zn⁺⁺), and 2-way ANOVA to test their interactions. P<0.05 was considered statistically significant.
RESULTS

TAC Induced LV Hypertrophy in All Groups.

There were no significant differences in body weight among groups. There were significant increases in heart weight in both the genotypes treated with either ZnSO₄ or H₂O at all time points following TAC. Both genotypes demonstrated cardiac hypertrophy, as evidenced by increased total heart and individual chamber weights as early as 7 days after TAC (Table). Similarly, cross sectional areas of CMs from LV of all genotype treatment groups were significantly greater at 28 days post TAC compared to sham controls (Table). RV weights were unchanged following TAC in all groups. Survival rates were 80% in the first 5 days post surgery, then 100% for the remainder of the study in all groups.

Normalized LV weight did not differ between genotypes after sham surgery and was increased in all mice following TAC. There were no significant differences in LV weight of DnTGFβRII+Zn⁺⁺ mice compared with the other 3 groups at any time point following TAC.

TAC-Induced Non-myocyte Proliferation in LV was Attenuated in DnTGFβRII+Zn⁺⁺ Mice

Non-myocyte proliferation assessed by Ki67 staining at 7 days post TAC was increased in the LV posterior wall and septum of NTG+H₂O, NTG+Zn⁺⁺ and DnTGFβRII+H₂O mice (Figures 1 and 2). In contrast, TAC-induced non-myocyte proliferation in LV of DnTGFβRII+Zn⁺⁺ was dramatically reduced, indicating that blocking TGF-β signaling attenuated non-myocyte proliferation. Sham operated
mice from all 4 genotype/treatment groups showed minimal non-myocyte proliferation in posterior wall and septum of LV (Figures 1 and 2).

**Apoptosis Was Minimal in All Groups**

TUNEL staining revealed very few apoptotic cells in any of the LV specimens from the 8 experimental groups (data not shown), suggesting that TAC-induced pressure overload stress did not increase apoptotic cell death in heart at 7 days after TAC.

**TAC-Induced Increase in Collagen Deposition in LV Was Attenuated in DnTGF\(\beta\)RII+Zn\(^{++}\) Mice**

At 28 and 120 days post TAC, NTG+H\(_2\)O, NTG+Zn\(^{++}\), and DnTGF\(\beta\)RII+H\(_2\)O mice developed 3 fold increases in total collagen protein content in LV compared with their sham operated controls (Figures 3 and 4). This effect appeared to be maximal at 28 days. Interstitial collagen levels were not significantly changed from sham control levels in LV of DnTGF\(\beta\)RII+Zn\(^{++}\) mice at either time point post TAC. There was no significant increase in % LV collagen volume in TAC-DnTGF\(\beta\)RII+Zn\(^{++}\) at 28 or 120 days after TAC compared with their sham operated controls (Figure 4). These results indicate that TGF-\(\beta\) signaling is necessary for interstitial collagen deposition in response to pressure-overload stress.

**TAC-Induced LV Dilation and Dysfunction but Not LV Hypertrophy Were Exacerbated in DnTGF\(\beta\)RII+Zn\(^{++}\) Mice**

Echocardiographic examination of the 4 TAC groups and sham treated NTG+H\(_2\)O mice was carried out at 120 days post TAC or sham surgery (Figures
LVEDD and EDV were not changed in NTG mice, but were increased in both groups of DnTGFβRII mice 120 days after TAC. The TAC-induced increases in LVEDD and EDV were significantly greater in DnTGFβRII+Zn²⁺ mice compared to their H₂O controls. LVESD and ESV were significantly increased in all genotype/treatment groups after TAC. Similar to LVEDD and EDV, the TAC-induced increases in LVESD and ESV were significantly greater in DnTGFβRII+Zn²⁺ mice. EF and FS did not differ between genotypes after sham surgery and were reduced in both genotypes after TAC. The TAC-induced decreases in EF and FS were significantly exacerbated by ZnSO₄ treatment in DnTGFβRII mice. CO and HR were not different among experimental groups.
DISCUSSION

In the present study, we have utilized a novel DnTGFBRRII mouse model of inducible inhibition of the TGF-β/Smad signaling cascade to define the contribution of TGF-β signaling to the phenotype of cardiac remodeling and fibrosis in response to pressure overload. The DnTGFβRII mouse model offers important advantages for studying the contribution of TGF-β signaling to pressure overload-induced cardiac hypertrophy and remodeling. TGF-β signaling is essential for the embryonic development of the heart, and homozygous deletion of the TGF-β gene leads to embryonic lethality, which has prevented the successful development of a TGF-β knock-out model (18). The mutation in the DnTGFβRII mouse can be induced in the mature animal, thus does not disrupt critical TGF-β signaling pathways in the developing heart, while providing the ability to selectively inhibit the downstream signaling of TGF-β at the receptor level later in life. Also, there is no need to repeatedly inject agents into the mice or monitor crests and troughs of drug levels. Our transgenic model works on the genomic level, rendering it superior to models that require pharmacologic interventions.

The major findings of this study are that under pressure overload stress, inhibition of TGF-β signaling results in dramatic reductions in non-myocyte proliferation and collagen content and subsequent development of LV dilation and systolic dysfunction in DnTGFβRII mice. It is well established that under stress the non-myocyte cells in the LV produce ECM proteins to ‘control’ the amount of remodeling in the overloaded ventricle (20,3,5). These proliferating
interstitial cells have been characterized as cardiac myofibroblasts by \( \alpha \)-smooth muscle actin staining in our previous study (20). Our current findings are consistent with our previous observations that TGF-\( \beta \) stimulates proliferation and myofibroblast transformation of isolated mouse cardiac fibroblasts (20) and that non-myocyte proliferation and ECM deposition account for pressure overload-induced LV enlargement in mice subjected to TAC (36). In the current study, there was minimal apoptotic staining (TUNEL stain) of either fibroblasts or myocytes in LV of all experimental groups after seven days of pressure overload, indicating that apoptosis does not play a major role in the LV remodeling observed in these animals.

In the current study, inhibition of the TGF\( \beta \)/Smad signaling pathway attenuated the deposition of collagen in our pressure overload model. In contrast to the other 3 treatment groups, there was no evidence of pressure overload-related increases in LV collagen levels in DnTGF\( \beta \)RII+Zn\( ^{++} \) hearts at any time point post TAC. By 120 days of pressure overload, there was evidence of exaggerated LV dilation and systolic dysfunction, as indicated by increased LV ESV and LVEDD, LVEDV and LVEDD, and decreased FS and EF by echocardiographic examination in DnTGF\( \beta \)RII+Zn\( ^{++} \) mice. Taken together, these data suggest that interstitial collagen maybe required to maintain ventricular structure under chronic pressure overload stress. This study adds to a growing body of knowledge concerning the role of TGF-\( \beta \) signaling in the pathogenesis of cardiac remodeling/fibrosis in response to various forms of stress in mouse models. A previous study in C57/BL6 mice subjected to TAC demonstrated
marked increases in TGF-β expression (mRNA for TGF-β1, -β2, -β3) and signaling (pSmad2 and pSmad1 protein levels) (37). At 3 days post TAC, the hearts exhibited marked concentric hypertrophy, but had no impairment in systolic function by echocardiography. However, after 28 days of TAC the LV was dilated and FS was reduced and collagen deposition significantly increased, indicating the development of heart failure and remodeling in the presence of increased TGF-β expression/signaling (37). This study provided a platform for our investigation of the role of TGF-β signaling in the pathogenesis of pressure overload-induced cardiac pathology.

Several recent studies using a myocardial infarction model have examined the importance of the TGF-β signaling pathway in determining functional and structural adaptations to ischemic cardiac injury. Inhibition of TGF-β signaling with a nonselective TGFβ1, 2, 3 inhibitor (1D11) has been shown to have detrimental effects on the structure and function of the LV of C57BL/6J mice with myocardial infarction induced by coronary artery ligation (14). Mice underwent intraperitoneal injection of 1D11 or control vehicle every other day beginning 1 week prior to or 5 days after coronary artery ligation. Survival was significantly reduced in both groups of 1D11 treated mice, (<50%) compared to the vehicle treated control mice (81%). Deaths were attributed to heart failure. Collagen 1 mRNA levels were attenuated at 3 days after ligation in the 1D11 pre treated group vs. control group. In contrast, at 8 wks collagen deposition, assessed by immunohistochemistry was unchanged in both infarct and noninfarct regions by 1D11 treatment compared to control. However, there was significantly increased
LV dilation over time (7, 21, 56 day echo) and a non significant worsening of fractional shortening in both 1D11 groups, consistent with our present findings (14). The cardiac effects of a novel oral selective TGFβRI antagonist (SD-208) have also been examined in a murine coronary artery ligation model (10). All mice survived the 30 day experimental period, during which SD-208 treatment reduced collagen deposition and cardiac mass in comparison to controls, supporting the importance of TGF-β signaling in cardiac remodeling.

Other studies have used adenoviral overexpression of the extracellular domain of the human TGFβRII to block TGF-β signaling in mouse infarct models (17,27). Administration of the TGFβRII plasmid 7 days before coronary ligation was associated with increased mortality and LV dilation, but when the same plasmid was administered on the day of ligation and 7 days later, there was significant attenuation of LV remodeling and dysfunction compared to controls (17). Similarly, in another study, administration of an adenoviral TGFβRII plasmid 3 days after coronary ligation was associated with attenuation of LV dilation and reduced cardiac fibrosis compared to controls (27). In contrast, administration of the plasmid 4 weeks after coronary ligation, after a scar had already formed, had no effect on LV remodeling. These findings suggest that activation of TGF-β signaling appears to be protective during early ischemic myocardial damage, but not during the late phase after MI. Thus, timing of interventions that inhibit TGF-β signaling is critical when evaluating differing results. Collectively, in infarct models, TGF-β antagonists reduce collagen deposition and cause varying degrees of cardiac dysfunction depending on the
time course and dose of treatment and time points that are evaluated. Early treatments appear to have protective effects on cardiac function, whereas later treatments have detrimental effects.

The effects of the oral TGFβRI antagonist NP-40208 on transgenic mice with cardiac-restricted overexpression of tumor necrosis factor (MHCsTNF) have also been examined (30). MHCsTNF mice develop a heart failure phenotype characterized by progressive myocardial fibrosis, an increase in TGF-β levels, and LV diastolic dysfunction as shown by an increase in the chamber stiffness constant using the Langendorff method. In this study, transgenic and control mice aged 4-12 wks were treated with NP-40208 for 4-12 wks, followed by harvesting of their hearts. Nuclear Smad 2/3 increased in the MHCsTNF group compared to littermate control mice, consistent with TGF-β activation. This effect was attenuated by administration of NP-40208, indicating TGF-β signaling blockade. MHCsTNF mice treated with NP-40208 had a small but statistically significant decrease in heart weight to body weight ratio and significantly attenuated LV collagen deposition compared to MHCsTNF control mice, as shown by picrosirius red staining, confirming that the TGF-β pathway plays an important role in the development of myocardial fibrosis.

The current findings complement our previous observations that the anti-fibrotic hormone atrial natriuretic peptide (ANP) is upregulated in heart under pressure or volume overload stress and is functionally active in modulating increases in fibrosis and remodeling. Mice with homozygous deletion of the pro-ANP gene (Nppa−/−) exhibit cardiac fibrosis under resting conditions (11,13, 26,36).
and develop exaggerated fibrosis and remodeling after pressure or volume overload compared to wild type control mice (13,36,26). These abnormalities appear in attenuated form in heterozygous Nppa+/− mice subjected to pressure overload stress resulting from TAC, indicating that even partial ANP deficiency results in adverse cardiac remodeling under hemodynamic stress (13). The profibrogenic phenotype that results from eliminating the anti-fibrotic ANP/cGMP/PKG signaling pathway appears to be related to unopposed TGF-β signaling in response to pressure overloaded stress. Our laboratory has shown that ANP and TGF-β play important counterregulatory roles in pressure overload-induced cardiac remodeling and fibrosis (12,13,24,36). We have also reported previously a direct interaction between the ANP/cGMP/PKG (anti-fibrotic) and TGF-β (pro-fibrotic) pathways in cardiac fibroblasts (20). TGF-β accelerates myofibroblast transformation and ECM production in cardiac fibroblasts and activation of the ANP signaling cascade inhibits these processes by interrupting TGF-β1 signaling at the level of Smad3 phosphorylation.

In summary, we observed that under pressure overload stress, inhibition of TGF-β signaling attenuates interstitial collagen content and myofibroblast proliferation, leading to LV dilation and dysfunction over a 4 month follow up period. These findings underscore the importance of the TGF-β signaling pathway in the pressure overloaded mouse heart, and demonstrate that blockade of this signaling pathway in our novel DnTGFβRII mouse model results in cardiac pathology by promoting LV dilation and dysfunction. Future studies of TGF-β signaling in the stressed heart are warranted to achieve a better understanding of
the molecular pathogenesis of the injury response with the ultimate goal of developing novel therapeutic strategies.
ACKNOWLEDGMENTS

This work was supported, in part, by National Heart, Lung, and Blood Institute grants; HL080017, HL044195 (Chen YF), HL07457, HL75211 (Oparil S), Cardiovascular Pathophysiology Predoctoral Training Grant; T32 HL007918, and by American Heart Association Greater Southeast Affiliate grants 0455197B (Chen YF), 0425455B, 0765398B (Xing D).
REFERENCES


increases mortality and left ventricular dilatation after myocardial infarction. 

*Basic Res Cardiol.* Sep;103(5):485-92. 2008


19. **Li P, Oparil S, Novak L, Cao X, Shi W, Lucas JA, Chen YF.** ANP Signaling Inhibits TGF-β-induced Smad2 and Smad3 nuclear translocation


7 days

Figure 1. Representative light micrographs of left ventricle (LV) from DnTGFβRII and NTG mice drinking 25 mM ZnSO4 water (Zn++) or double distilled water (H2O) 7 days after transverse aortic constriction (TAC) or sham (Control) operation. Cross sections of middle circular layer of posterior wall were immunostained with selective anti-nuclear Ki67 antibody (brown color). Arrows indicate representative positive signals localizing the interstitial non-myocytes. Magnification 400X.
Figure 2. Effects of 7 days of TAC or sham operation (Control) on density of Ki-67 positive interstitial non-myocytes in (A) posterior wall and (B) septum of DnTGFβRII and NTG mice drinking 25 mM ZnSO4 water (Zn++) or distilled water (H2O). Six 400X cross section areas of posterior wall and six septal areas per mouse were measured and averaged. Results are means±SEM; (n) = number of mice per group. * p<0.05 compared with respective Control groups; # p<0.05 compared with respective TAC-H2O groups by ANOVA.
Figure 3. Representative picrosirius red-stained cross sections at a level below the mitral valve of posterior wall of DnTGFβRII and NTG mice drinking 25 mM ZnSO4 water (Zn++) or distilled water (H2O) 28 and 120 days after TAC. Controls were sham operated mice without TAC and sacrificed at 14-16 wk of age. Magnification 400X.
Figure 4. Effects of 28 and 120 days of TAC on interstitial collagen volume (picrosirius red-stained areas) in LV of DnTGFβRII and NTG mice drinking 25 mM ZnSO4 water (Zn++) or distilled water (H2O). Six 400X cross section areas of posterior wall and six septal areas per mouse were measured and averaged. Controls are mice without TAC and sacrificed at 14-16 wk of age. Results are means ±SEM; (n) = number of mice per group. * p<0.05 compared with respective sham control groups; # p<0.05 compared with respective TAC-H2O groups by ANOVA.
Figure 5. Representative micrographs of 2-D guided M-Mode echocardiography of Sham-NTG+H2O, TAC-NTG+H2O, and TAC-DnTGRβRII+Zn++ mice at 120 days after TAC or sham operation.
Figure 6. Effects of 120 days of TAC on (A) body weight (BW), (B) LV/BW ratio, (C) left ventricular end diastolic dimension (LVEDD), (D) left ventricular end systolic dimension (LVESD), (E) end diastolic volume (EDV), (F) end systolic volume (ESV), (G) ejection fraction (EF), and (H) fractional shortening (FS) in DnTGFβRII and NTG mice drinking 25 mM ZnSO4 water (Zn++) or distilled water (H2O). Controls are mice drinking H2O and sacrificed at the same age as TAC mice. The EDV, ESV, EF and FS were assessed by echocardiography and adjusted by analysis of covariance (ANOVA) with BW as a covariate. Results are mean±SEM, (n) = number of mice per group. * p<0.05 compared with Control groups; # p<0.05 compared with respective H2O-TAC groups; Δ p<0.05 compared with respective NTG groups by ANOVA.
Table. Effects of 7, 28, and 120 days of TAC and/or 25 mM ZnSO₄ in drinking water on left (LV) and right (RV) ventricular weights and LV cardiomyocyte (CM) size of DnTGFβRII and NTG mice.

<table>
<thead>
<tr>
<th></th>
<th>NTG H₂O Sham</th>
<th>NTG ZnSO₄ Sham</th>
<th>NTG H₂O TAC</th>
<th>NTG ZnSO₄ TAC</th>
<th>DnTGF βRII H₂O Sham</th>
<th>DnTGF βRII ZnSO₄ Sham</th>
<th>DnTGF βRII H₂O TAC</th>
<th>DnTGF βRII ZnSO₄ TAC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>7 days after TAC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BW (g)</strong></td>
<td>27.6±0.6</td>
<td>26.0±0.7</td>
<td>31.1±0.7</td>
<td>27.6±0.7</td>
<td>27.0±0.6</td>
<td>26.6±0.5</td>
<td>26.1±0.8</td>
<td>27.1±1.0</td>
</tr>
<tr>
<td><strong>LV (mg)</strong></td>
<td>90.6±2.5</td>
<td>86.4±3.3</td>
<td>133.8±7.5*</td>
<td>117.6±5.3*</td>
<td>98.6±1.8</td>
<td>98.4±2</td>
<td>117.6±8.5*</td>
<td>123.1±5.5*</td>
</tr>
<tr>
<td><strong>RV (mg)</strong></td>
<td>16.9±0.8</td>
<td>16.9±0.8</td>
<td>19.9±0.8</td>
<td>16.4±1.2</td>
<td>16.8±1</td>
<td>17.8±1.2</td>
<td>16.4±0.8</td>
<td></td>
</tr>
<tr>
<td><strong>28 days after TAC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BW (g)</strong></td>
<td>29.6±0.4</td>
<td>33.0±2.5</td>
<td>28.8±0.8</td>
<td>28.9±0.9</td>
<td>28.1±0.7</td>
<td>26.5±0.6</td>
<td>23.0±0.6</td>
<td>23.4±1.1</td>
</tr>
<tr>
<td><strong>LV (mg)</strong></td>
<td>99.7±2.5</td>
<td>90.5±3.6</td>
<td>137.1±8.1*</td>
<td>137.7±3.7*</td>
<td>91.0±2.9</td>
<td>91.9±2.0</td>
<td>112.6±5.6*</td>
<td>117.6±5.7*</td>
</tr>
<tr>
<td><strong>RV (mg)</strong></td>
<td>26.3±2.3</td>
<td>22.6±2.1</td>
<td>22.3±1.6</td>
<td>22.1±0.8</td>
<td>22.6±0.4</td>
<td>23.0±1.4</td>
<td>19.1±0.7</td>
<td>18.8±1.4</td>
</tr>
<tr>
<td><strong>CM Area (μm²)</strong></td>
<td>111±5</td>
<td>124±8</td>
<td>175±6*</td>
<td>178±11*</td>
<td>133±6</td>
<td>123±5</td>
<td>172±4*</td>
<td>176±7*</td>
</tr>
<tr>
<td><strong>120 days after TAC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BW (g)</strong></td>
<td>30.6±0.5</td>
<td>-</td>
<td>36.1±1.9</td>
<td>33.1±2.2</td>
<td>30.3±0.8</td>
<td>-</td>
<td>32.8±2</td>
<td>31.2±1.4</td>
</tr>
<tr>
<td><strong>LV (mg)</strong></td>
<td>94±3</td>
<td>-</td>
<td>184±16*</td>
<td>195±16*</td>
<td>103±3</td>
<td>-</td>
<td>164±16*</td>
<td>187±15*</td>
</tr>
<tr>
<td><strong>RV (mg)</strong></td>
<td>27±2</td>
<td>-</td>
<td>39±9</td>
<td>39±8</td>
<td>33±3</td>
<td>-</td>
<td>32±3</td>
<td>28±3</td>
</tr>
</tbody>
</table>

Results are means±SEM. The normalized LV and RV weights were determined by analysis of covariance (ANCOVA) with body weight (BW) as a covariate. *p<0.05 compared with respective sham control groups by 2-way ANOVA.
ANP SIGNALING INHIBITS TGF-B INDUCED SMAD2 AND SMAD3 NUCLEAR TRANSLOCATION AND EXTRACELLULAR MATRIX EXPRESSION IN RAT PULMONARY ARTERIAL SMOOTH MUSCLE CELLS

by

PENG LI, SUZANNE OPARIL, LEA NOVAK, XU CAO, WEIBIN SHI,

JASON LUCAS, YIU-FAI CHEN


Copyright 2007 by American Physiological Society.

Used by permission.

Format adapted for dissertation

- 49 -
ABSTRACT

Atrial natriuretic peptide (ANP) and transforming growth factor (TGF)-β play important counterregulatory roles in pulmonary vascular adaptation to chronic hypoxia. To define the molecular mechanism of this important interaction, we tested whether ANP-cGMP-PKG signaling inhibits TGF-β1-induced extracellular matrix (ECM) expression and defined the specific site(s) at which this molecular merging of signaling pathways occurs. Rat pulmonary arterial smooth muscle cells (PASMCs) were treated with ANP (1 µM) or cGMP (1 mM) with or without pretreatment with the PKG inhibitors KT5823 (1 µM) or Rp-8-Br-cGMP (50 µM), then exposed to TGF-β1 (1 ng/ml) for 5-360 min (for pSmad nuclear translocation and protein analysis) or 24 hrs (for ECM mRNA expression). Nuclear translocation of pSmad2 and pSmad3 was assessed by fluorescent confocal microscopy. ANP and cGMP inhibited TGF-β1-induced pSmad2 and pSmad3 nuclear translocation and expression of periostin (PN), osteopontin (OPN) and plasminogen activator inhibitor (PAI)-1 mRNA and protein, but not TGF-β1-induced phosphorylation of Smad2 and Smad3. KT5823 and Rp-8-Br-cGMP blocked ANP/cGMP-induced activation of PKG and inhibition of TGF-β1-stimulated nuclear translocation of pSmad2 and pSmad3 in PASMCs. These results reveal for the first time a precise site at which ANP-cGMP-PKG signaling exerts its anti-fibrogenic effect on the pro-fibrogenic TGF-β1 signaling pathway: by blocking TGF-β1-induced pSmad2 and pSmad3 nuclear translocation and ECM expression in PASMCs. Blocking nuclear translocation and subsequent binding of pSmad2 and pSmad3 to TGF-β-Smad response
elements in ECM genes may be responsible for the inhibitory effects of ANP on TGF-β-induced expression of ECM molecules.

**Key Words:** Lung, Vascular hypertrophy and remodeling, Pulmonary arterial smooth muscle cells, Atrial natriuretic factor, Transforming growth factor, Signal transduction, Extracellular Matrix.
INTRODUCTION

Hypoxia-induced pulmonary hypertension and vascular remodeling result from an imbalance in the normal relationships between vasoconstrictor and vasodilator and between mitogenic and growth inhibiting pathways in the pulmonary vasculature. Our previous studies in rodent models have provided convincing evidence that endogenous atrial natriuretic peptide (ANP) and transforming growth factor (TGF)-β play important counterregulatory roles in pulmonary vascular adaptation to chronic hypoxia (3,29). ANP and TGF-β expression are upregulated in heart and lung, respectively, of hypoxia adapted animals (1,2,26,29). The functional significance of the hypoxia-induced increase in ANP is supported by observations of worsening pulmonary hypertension and vascular remodeling in ANP deficient (Nppa-/−) mice compared to wild type controls in response to hypoxic stress (29). Our recent studies in a novel mouse model that expresses an inducible dominant negative mutation of the TGF-β receptor type II gene (DnTGFβRII), and thus cannot activate the TGF-β-Smad signaling cascade, demonstrate that disruption of TGF-β signaling greatly attenuates hypoxia-induced pulmonary hypertension, right ventricular hypertrophy, pulmonary arterial remodeling and muscularization, and expression of extracellular matrix (ECM) in lung, supporting a critical role for TGF-β signaling in the pathogenesis of chronic hypoxia-induced pulmonary hypertension and vascular remodeling (3).

ANP, via activation of guanylate cyclase-coupled membrane receptors, increases intracellular cGMP levels and activates cGMP-dependent protein
kinase (PKG), with resultant growth inhibiting and antiproliferative effects in a variety of cell types, including pulmonary arterial smooth muscle cells (PASMCs) (2,10,22). In contrast, activated TGF-β participates in pulmonary morphogenesis and in the pathogenesis of pulmonary fibrosis and vascular remodeling by stimulating PASMC proliferation and ECM expression (1,23,33). TGF-β signals through membrane bound heteromeric type I (TGFβRI) and type II (TGFβRII) receptor kinases that transduce intracellular signals via phosphorylation and nuclear translocation of receptor-activated Smad2 and Smad3 proteins, which modulate the transcription of a large number of genes (20,28). The molecular mechanisms of the counterregulatory effects of ANP-cGMP-PKG signaling on activated TGF-β-induced Smad signaling in PAMSCs have not been studied.

In the present study, we tested the hypothesis that ANP signaling inhibits TGF-β1-stimulated ECM expression in isolated PASMCs and probed the specific site(s) at which molecular merging of these pro- and anti-fibrogenic signaling cascades occurs. Specifically, we tested whether ANP-cGMP-PKG signaling interrupts downstream events in the TGF-β1 signaling pathway by inhibiting TGF-β1-induced phosphorylation of Smad2 and Smad3 proteins and/or preventing nuclear translocation of phosphorylated Smad2 and Smad3.
MATERIALS AND METHODS

Animals and cell culture

Young adult (8 wk old) male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were used. All experimental protocols were approved by the University of Alabama at Birmingham (UAB) IACUC Committee and were consistent with the NIH Guide for the Care and Use of Laboratory Animals (DHEW Publication No. 96-01, 1996).

PASMCs were isolated from distal segments of pulmonary arteries (2nd-3rd branches, 0.1-0.2 mm external diameter) using the explant method described previously (14,15,30). To confirm the characteristics of smooth muscle cells (SMCs) in culture, immunohistochemical staining of α-SMC actin was performed using selective α-SMC actin antibody and secondary horse radish peroxidase-labeled anti-IgG antibodies. All cultures were examined by phase contrast microscopy before and after each experimental period to assess cell viability. PASMCs were used for experiments at passage 3 or 4.

Prior to each study, PASMCs were grown in 10% fetal bovine serum (FBS)-DMEM to 95% confluence and then made quiescent by placing them in medium containing 0.1% FBS for 24 hrs. For Northern, real-time quantitative RT-PCR or Western blot analyses, PASMCs were cultured in 60-mm cell culture dishes. For quantitative analysis of nuclear translocation of phosphorylated Smad2 (pSmad2) and Smad3 (pSmad3), PASMCs were cultured on 18 mm x 18 mm glass cover slides.
Experimental protocols

**Experiment 1: Effects of ANP signaling on TGF-β1-stimulated ECM expression**

To test the hypothesis that ANP and cGMP inhibit TGF-β1-stimulated expression of mRNA for the extracellular matrix (ECM) proteins periostin (PN), osteopontin (OPN) and plasminogen activator inhibitor 1 (PAI-1, a biomarker of TGF-β1 action in cells), quiescent PASMCs were treated with TGF-β1 (0.1 to 5 ng/ml) (Sigma) for 24 hrs with or without pretreatment with ANP (1 µM) (Sigma), cGMP (8-Br-cGMP, 1 mM) (Sigma), and/or PKG inhibitors KT5823 (1 µM) (CalBiochem) or Rp-8-Br-cGMP (50 µM) (Alexis) for 30 min. PASMCs were then harvested for Northern blot, real time quantitative RT-PCR, or Western blot analyses.

**Experiment 2: Effects of TGF-β1 and cGMP on phosphorylation of Smad3 and Smad2 proteins in PASMCs**

To test the hypothesis that cGMP inhibits TGF-β1-induced phosphorylation of Smad2 and Smad3 in PASMCs, quiescent PASMCs were pretreated with cGMP (0.01 to 1 mM) or vehicle for 30 min before addition of TGF-β1 (1 ng/ml) to the medium and incubation for an additional 30 min. After treatment with TGF-β1 and/or cGMP, cells were harvested for assessment of pSmad2, pSmad3 and Smad4 (internal control that cannot be phosphorylated) levels using Western blot analysis.
Experiment 3: Effects of ANP/cGMP on TGF-β1-induced nuclear translocation of pSmad2 and pSmad3 in rat PASMCs

To test the hypothesis that inhibition of TGF-β1-stimulated ECM expression by ANP-cGMP-PKG signaling is dependent on events downstream from phosphorylation of Smad2 and Smad3, we examined the effects of ANP and cGMP on TGF-β1-stimulated nuclear translocation of pSmad2 and pSmad3 in PASMCs. Quiescent rat PASMCs were pretreated with ANP (1 µM), cGMP (1 mM) or vehicle for 30 min, and then exposed to TGF-β1 (1 ng/ml) for an additional 5 min to 6 hr. PASMCs were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X100 in PBS. The fixed PASMCs were stained with selective anti-pSmad2, anti-pSmad3, and anti-Smad4 primary antibodies and a Texas Red-labeled donkey anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Lab) to assess nuclear translocation of pSmad2 and pSmad3 using confocal fluorescence microscopy with a computerized Zeiss-Axioskop system.

Experiment 4: Effects of ANP and cGMP on PKG activity in rat PASMCs

To test the hypothesis that ANP and/or cGMP activates PKG in rat PASMCs, quiescent PASMCs were pretreated with PKG inhibitors KT5823 (1 µM) or Rp-8-Br-cGMP (50 µM), or vehicle for 15 min before adding ANP (1 µM) or cGMP (1 mM). Fifteen min after beginning treatment with ANP or cGMP, cells were exposed to TGF-β1 (1 ng/ml) for an additional 15 min. Cells were then harvested for PKG activity measurement.
Experiment 5: ANP/cGMP signaling inhibits TGF-β1-induced nuclear translocation of pSmad2 and pSmad3 in rat PASMCs by activating PKG in rat PASMCs.

To test the hypothesis that ANP/cGMP signaling inhibits TGF-β1-induced nuclear translocation of pSmad3 via activation of PKG, quiescent PASMCs cultured on slides were pretreated with PKG inhibitors KT5823 (1 µM) or Rp-8-Br-cGMP (50 µM), or vehicle for 15 min before adding ANP (1 µM) or cGMP (1 mM). Cells were incubated with TGF-β1 for an additional 60 min, then fixed and stained with selective anti-pSmad3, as in Experiment 3. Nuclear translocation of pSmad3 was assessed using confocal fluorescence microscopy, as in Experiment 3.

RNA isolation for Northern blot

PASMC were homogenized, and total RNA was extracted using the TRIZOL total RNA isolation reagent (Invitrogen Corp). Northern analysis was performed using a ³²P-labeled selective cDNA probes for PN, OPN, PAI-1 and GAPDH that had been generated in our laboratory by reverse transcription (RT) followed by the DNA polymerase chain reaction (PCR) using lung RNA as the template, as previously described (14,15). A ³²P-labeled 18S rRNA-oligonucleotide (5'-ACGGTATCTGATCGTCTTCGAACC-3') was used as the control probe to normalize data. Autoradiographic signals were scanned with an optical densitometer (Bio-Rad, Model GS-670 Imaging Densitometer). To estimate steady-state specific mRNA levels, PN and OPN mRNA/18S rRNA and PAI-1 mRNA/GAPDH mRNA ratios were determined by dividing the absorbance
corresponding to the specific cDNA probe hybridization by the absorbance corresponding to the 18S rRNA or GAPDH cDNA probe hybridization.

**Western blot analysis**

Standard Western blot analysis for phosphor-Smad2, phosphor-Smad3, Smad4, periostin, vasodilator-stimulated phosphoprotein (VASP, a substrate of PKG), phosphor-VASP (p-VASP [ser239], a selective product of PKG), periostin, osteopontin, and β-actin was performed using anti-pSmad2, anti-pSmad3, anti-Smad4, anti-Smad2/3, anti-VASP, anti-pVASP (Cell Signaling), anti-periostin (Abcam), and anti-osteopontin (a gift from Dr. P-L Chang, 9) specific primary antibodies and a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG. Immune complexes were detected using a Phototop-HRP Western Detection Kit (Cell Signaling). Autoradiograms exposed in the linear range of film density were scanned using a densitometer (Bio-Rad Model GS-670 Imaging Densitometer) as described previously (14,15).

**PKG activity assay**

PASMC lysates were assayed for PKG activity by measuring 1) the incorporation of $^{32}$P from $[^{32}$P] ATP into a specific PKG substrate, Glasstide (CalBiochem) using a modified method of Lincoln et al. (17) and 2) the phosphorylation at Ser239 of VASP (Cell Signaling) using a modified method of Lawrence et al. (12) and Li et al. (16). To measure the PKG-stimulated incorporation of $^{32}$P to Glasstide, PASMCs were lysed in CellLytic™-M Lysis Reagent (Sigma) plus 10 mM dithiothreitol, 1 mM isobutylmethylxanthine, 1X Halt™ Protease inhibitor Cocktail (Pierce). Lysates were sonicated and
centrifuged at 14,000 rpm for 15 min at 4°C and supernatants were assayed for PKG activity without adding exogenous cGMP. PKG activity was normalized to the protein concentration of the supernatant, measured by Bradford's procedure, using BSA as a standard. To measure the PKG-stimulated Ser239 phosphorylation of VASP, PASMCs were lysed in 1X SDS buffer (2% SDS, 10% glycerol, 50 mM DTT, 0.01% bromophenol blue in 62.5 mM Tris-HCl, pH 6.8). Lysates were sonicated and subjected to Western blot analysis for quantitation of VASP and p-Ser239-VASP (Cell Signaling).

**Statistical analysis**

Results were expressed as means ± SEM. Statistical analyses were carried out using the SigmaStat package (Jandel Scientific Software, San Rafael, CA) on a PC. Statistical comparisons of mRNA levels were performed with the one-way analysis of variance (ANOVA) or unpaired t-test. If ANOVA results were significant, a post-hoc comparison among groups was performed with the Newman-Keuls test. Differences were reported as significant if the P value was < 0.05.
RESULTS

ANP and cGMP inhibit TGF-β1-stimulated PN, OPN and PAI-1 expression in PASMCs

Northern blot analysis demonstrated that PN and OPN mRNA expression increased in a dose-dependent fashion in PASMCs treated with TGF-β1 for 24 hrs (Figures 1A and 1B). The threshold concentration was between 0.1 and 1 ng/ml, and the maximum effect was observed at doses of 5 ng/ml for PN (a 3.3-fold increase) and OPN (a 3.7-fold increase), respectively. Pretreatment with ANP (1 μM) or cGMP (1 mM) decreased baseline levels of PN and OPN and significantly attenuated TGF-β1 (1 ng/ml)-induced expression of PN and OPN mRNAs (Figures 1C and 1D). Western blot analysis confirmed that pretreatment of cGMP (1 mM) blocked TGF-β1 (1 ng/ml)-induced PN and OPN protein expression (Figure 1F).

Similarly, pretreatment with cGMP (1 mM for 30 min) blocked TGF-β1 (1 ng/ml for an additional 24 hrs)-stimulated PAI-1 mRNA expression, assessed by real-time quantitative RT-PCR (Figure 1E). Pretreatment with the PKG inhibitors KT5823 (1 μM) or Rp-8-Br-cGMP (50 μM) blocked the inhibitory effects of cGMP on TGF-β1-stimulated PAI-1 expression in PASMCs, suggesting that the action of cGMP was through the activation of PKG. These data support the hypothesis that ANP-cGMP-PKG signaling has anti-fibrogenic effects that antagonize TGF-β-induced stimulation of ECM expression in PASMC.
cGMP does not inhibit TGF-β1-induced phosphorylation of Smad3 and Smad2 in PASMCs

Western blot analysis demonstrated that TGF-β1 treatment significantly increased pSmad3 and pSmad2 levels in PASMCs and that pretreatment with cGMP did not inhibit TGF-β1-induced phosphorylation of Smad3 or Smad2 in these cells. Smad4 levels were not altered by either TGF-β1 or cGMP treatment (Figure 2A). Neither cGMP nor TGF-β1 treatment altered total Smad2/3 levels in these cells (Figure 2B). These results indicate that activation of ANP-cGMP signaling does not block TGF-β1-induced phosphorylation of Smad2 and Smad3 and thus that disruption of Smad2 and Smad3 phosphorylation does not account for the inhibitory effects of ANP and cGMP on TGF-β1-induced ECM expression.

ANP and cGMP inhibit TGF-β1-induced nuclear translocation of pSmad2 and pSmad3 in PASMCs

In vehicle treated cells, immunostaining of pSmad2 and pSmad3 was weak and distributed evenly in cytoplasm and nucleus, suggesting that pSmad2 and pSmad3 levels were low and without significant nuclear translocation (Figures 3 and 4). TGF-β1 treatment significantly stimulated nuclear translocation of pSmad2 and pSmad3, indicated by strong pSmad2 and pSmad3 staining in the nucleus. Pretreatment with ANP or cGMP significantly attenuated nuclear translocation of pSmad2 and pSmad3.

The number of cells with pSmad3 nuclear translocation and intensity of the pSmad3 signal in TGF-β1 (1 ng/ml for 30 min) treated PASMCs were
significantly greater than that of pSmad2 (Figures 5A and 5B). Pretreatment with ANP (1 µM for 30 min) or cGMP (1 mM for 30 min) blocked TGF-β1-induced nuclear accumulation of pSmad2 and pSmad3 (Figures 5A and 5B), with a substantial level of pSmad2 and Smad3 staining remaining in the cytoplasm in most cells (Figures 3 and 4).

When PASMCs were exposed to TGF-β1 (1 ng/ml) for 5 to 360 min, the nuclear accumulation of pSmad2 and pSmad3 began at 15 min, peaked at 30 min (pSmad2) and 60 min (pSmad3), and decreased but did not reach pretreatment levels by 3 and 6 hrs of TGF-β1 treatment (Figures 5C and 5D). The maximal percentages of PASMCs with pSmad2 and pSmad3 nuclear translocation were 23% and 94%, respectively. Pretreatment with cGMP (1 mM for 30 min) decreased both the peak and duration of TGF-β1-induced pSmad2 and pSmad3 nuclear translocation (Figures 5C and 5D).

**Inhibition of PKG attenuates the inhibitory effects of ANP and cGMP on TGF-β1-induced nuclear translocation of pSmad3 in PASMCs**

Both ANP (1 uM) and cGMP (1 mM) increased PKG activity in PASMCs within 15 min of treatment. Pretreatment with PKG inhibitors KT5823 or Rp-8-Br-cGMP blocked the ANP and cGMP-induced increases in PKG activity, and TGF-β1 did not alter cellular PKG activity in these cells (Figures 6A and 6B).

To test whether activation of PKG mediates the inhibitory effects of ANP and cGMP on TGF-β1-induced nuclear translocation of pSmads, pSmad3 nuclear translocation was measured in the presence of the PKG inhibitors KT5823 or Rp-8-Br-cGMP. Pretreatment with KT5823 or Rp-8-Br-cGMP blocked
the inhibitory effects of ANP and cGMP on TGF-β1-stimulated pSmad3 nuclear translocation (Figure 7).

**DISCUSSION**

In the present study, we demonstrate for the first time that ANP has an anti-fibrogenic effect on PASMCs treated with TGF-β1. ANP and cGMP suppressed TGF-β-stimulated ECM gene expression by interfering with Smad signaling through a PKG-dependent mechanism. The most striking finding of this study is that ANP and cGMP inhibit TGF-β1-induced nuclear translocation of pSmad2 and pSmad3, but not the TGF-β1-induced phosphorylation of Smad2 and Smad3 in PASMCs, thus defining a novel molecular mechanism by which ANP signaling intercepts the TGF-β signaling pathway and blocks TGF-β-induced ECM expression.

TGF-β is a key mediator of pulmonary morphogenesis and of the pathogenesis of pulmonary fibrosis and vascular remodeling (1,7,23,33). Increases in the local abundance of TGF-β1 promote vascular wall remodeling, arterial lesion growth, and vascular cell differentiation (25). Small amounts of TGF-β are present in a latent, inactive form in the normal adult lung, and expression of TGF-β is increased in pathological conditions, including cystic fibrosis, asthma, and hypoxia-induced pulmonary hypertension and vascular remodeling (1,6,26). TGF-β1 is involved in fibrotic tissue remodeling and is overexpressed in areas of active fibrosis in lung (4), as well as in several animal models of pulmonary hypertension (5,24).
Activated TGF-β ligands bind to a heteromeric complex of type II (TGFβRII) and type I (TGFβRI) receptors that transduce intracellular signals via activation of Smad2 and Smad3 (21,28). Phosphorylation of TGF receptor-associated Smad2 and Smad3 and nuclear translocation of pSmad2 and pSmad3 are critical steps in TGF-β signaling (28,32) (Figure 8). Smad2 (467 a.a.) and Smad3 (425 a.a.) contain predominantly serine, with some threonine residues, in the C-terminal, linker and MH1 regions that are accessible for phosphorylation. Upon ligand binding, phosphorylation by TGFβR1 kinase of the two most C-terminal serine residues drives the activation of Smad2 and Smad3 is required for nuclear translocation and subsequent binding of pSmads to nuclear transcriptional factors and DNA that regulate the transcriptional expression of downstream genes (28,32).

Input from other receptor systems, e.g. natriuretic peptide receptor type A (NPRA)-cGMP-PKG influences the Smad signaling network and the cross-talk created by the interplay between Smads and other signaling pathways is largely responsible for the diverse and context-specific effects of the TGF-β family of proteins (11,19,28). Both inhibition of phosphorylation and over-phosphorylation of Smad2 and Smad3, disruption of their heterodimerization with Smad4 and nuclear translocation, as well as inhibition of their binding to transcription factors and TGF-β responsive promoters on DNA have been reported to suppress TGF-β-stimulated gene expression (11,19,27,28,34). Thus, phosphorylation not only activates Smad proteins but also modulates their activity. This provides a novel mechanism for integration of the Smad pathway with ANP-NPRA-cGMP-PKG.
signaling that could modulate TGF-β signal transduction (Figure 8). The finding that ANP and cGMP inhibit TGF-induced nuclear translocation of pSmad2 and pSmad3, but not the TGF-β-stimulated phosphorylation of Smad2 and Smad3, in PASMCs suggests a novel molecular mechanism by which ANP-cGMP-PKG signaling intercepts the TGF-β signaling cascade. The results suggest that the PKG may act as a stronger protein kinase than TGFβRI and phosphorylate additional serine residues (i.e. in the linker or MH1 regions) on Smad2 and/or Smad3, thus disrupting their nuclear translocation, resulting in repression of transcriptional activation of TGF-β response promoters, e.g. on PN, OPN and PAI-1 genes.

We recognize that over-phosphorylation of Smads is not the only cellular function of PKG activation. PKG has diverse intracellular actions, including integrin signal transduction, modulation of Ca2+ release and uptake into sacroplasmic reticulum, alteration of membrane K+ fluxes, and nuclear protein phosphorylation and translocation (17). An alternative (to over-phosphorylation) explanation for the observation that ANP signaling prevents nuclear translocation of Smads is that cGMP-PKG may alter the affinity of Smads for cytoplasmic anchoring molecules or nuclear export proteins. Subcellular localization of Smads has been shown to be controlled by interaction with these cytoplasmic and nuclear retention factors (28). The precise molecular basis for retention of pSmads in the cytosol following ANP or cGMP treatment remains to be identified and is a topic for current investigation in our laboratory.
Several recent studies have demonstrated that various cytoplasmic protein kinases and cyclic nucleotides participate in regulating responses to TGF-β (18,19). In normal epithelial cells, Erk MAP kinase inhibits TGF-β signaling via inhibition of nuclear accumulation of Smad2 (11), but in malignant epithelial cells, Erk does not alter the function of Smad2, 3, or 4 at the level of nuclear translocation, DNA binding or transcriptional activation (13). Protein kinase C (PKC) directly phosphorylates Smad3 and abrogates the ability of Smad3 to bind directly to DNA, leading to impairment of transcriptional responses dependent on the direct binding of Smad3 to DNA (34). Specifically, PKC has been shown to block pro-apoptotic action of TGF-β in Mv1Lu mink lung epithelial cells. Activation of Ca++-calmodulin-dependent protein kinase II (Cam kinase II) prevents Smad2/4 heterodimerization and nuclear translocation and concomitant transcriptional responses in HEK-293 human kidney fibroblasts (31). Further, intracellular cAMP-elevating agents such as prostaglandin E2 (PGE2), the adenylate cyclase activator forskolin, and the phosphodiesterase inhibitor isobutyl-methylxanthine, inhibit TGF-β-induced Smad3/4-dependent gene expression via a cAMP-dependent protein kinase A (PKA)-dependent mechanism in human keratinocytes (27). Interestingly, activation of cAMP-PKA does not inhibit nuclear translocation and DNA binding of Smad3/4 complexes, but abolishes interactions of Smad3/4 with transcription activators in a PKA-dependent manner in the nucleus.

The current study shows that ANP and cGMP inhibit TGF-β-induced nuclear translocation of pSmad2 and pSmad3 in PASMCs, defining a new role for cyclic
mononucleotide phosphate second messenger in regulating pro-fibrogenic responses to TGF-β. Pretreatment with the PKG inhibitors KT5823 or Rp-8-Br-cGMP prevented the inhibitory effects of ANP and cGMP on TGF-β-stimulated nuclear translocation of pSmad2 and pSmad3, supporting the hypothesis that these effects are mediated through activation of PKG.

TGF-β mediates fibrotic tissue remodeling by increasing the production and decreasing the degradation of ECM (1,5,33). The present study is the first to demonstrate increased expression of PN, a novel ECM molecule originally described in bone (8), in PASMCs is part of a generalized ECM response to TGF-β. This finding, coupled with our previous observation of increased PN expression in lung of mice adapted to hypoxia (3), suggests an involvement of this novel ECM molecule in hypoxia-induced pulmonary vascular remodeling. Our finding that ANP and cGMP inhibit TGF-β1-induced expression of the ECM molecules PN and OPN, as well as PAI-1, a TGF-β-Smad target gene in PASMCs, indicates that there is a functionally significant interaction between ANP and TGF-β signaling that may play an important role in modulating hypoxia-induced pulmonary vascular remodeling.

Our previous in vivo studies have validated the importance of TGF-β and ANP as opposing influences in the pathogenesis of chronic hypoxia-induced pulmonary hypertension (3,29). Using a novel DnTGFβRII mouse model, we have demonstrated that hypoxia-induced pulmonary hypertension and vascular and parenchymal remodeling and right ventricular hypertrophy are markedly attenuated by disruption of TGF-β signaling in lung (3). In contrast, disruption of
ANP expression in ANP null mice exacerbates these hypoxia-induced processes (29). Taken together, these data support the hypothesis that endogenous TGF-β and ANP play important counterregulatory (ying-yang) roles in regulating pulmonary artery pressure, ECM production and pulmonary vascular remodeling in response to hypoxic stress. An imbalance in the normal relationships between the mitogenic/pro-fibrogenic of TGF-β and anti-growth/anti-fibrogenic effects of ANP results in chronic hypoxia-induced pulmonary hypertension and vascular and parenchymal remodeling.
ACKNOWLEDGMENTS

This work was supported in part by National Institute of Health Grants HL-44195, HL-64614, HL-075211, CA101955, DK60913, and an American Heart Association-0455197B Grant in Aid.

CONFLICT OF INTEREST DISCLOSURES

There is no conflict of interest to be disclosed.
REFERENCES


7. Evans SE, Colby TV, Ryu JH, Limper AH. Transforming growth factor-beta


Figure 1. (A & B) Dose-dependent stimulatory effects of TGF-β1 on steady-state periostin (PN) and osteopontin (OPN) mRNA expression in rat PASMCs. Quiescent PASMCs cultured in 0.1% FBS medium for 24-hr were exposed to graded doses of TGF-β1 or vehicle for an additional 24 hrs before being harvested. (C, D & E) Inhibitory effects of ANP or cGMP on TGF-β1-stimulated PN, OPN and PAI-1 mRNA expression in rat PASMCs. Quiescent PASMCs were pretreated with ANP (1 µM) or cGMP (8-Br-cGMP, a cGMP analog, 1 mM) for
30 min prior to TGF-β1 (1 ng/ml) for an additional 24 hrs before being harvested. In E), subgroups of PASMCs were pretreated with PKG inhibitors KT5823 (1 µM) or Rp-8-Br-cGMP (50 µM) for 15 min prior to ANP or cGMP. Numbers in parentheses are the numbers of plates contributing data to each group. Northern blot analysis was carried out with 15 µg of total RNA extracted from each plate. mRNA from each plate was quantitated individually. The Northern blot membrane was probed with PN and OPN cDNAs and 18S rRNA oligonucleotide or with PAI-1 and GAPDH cDNAs sequentially. PN and OPN mRNA data were normalized to the 18S rRNA and PAI-1 mRNA, to GAPDH mRNA to allow for variation in RNA loading. F) Cell lysates (25 µg) were size fractionated by SDS-PAGE, and Western blot analysis was performed with selective anti-PN, OPN, and β-actin antibodies. Results demonstrated that pretreatment with cGMP (1 mM for 30 min) blocked TGF-β1 (1 ng/ml for 24 hrs)-stimulated PN and OPN protein expression in rat PASMCs. Beta-actin protein levels were measured to show protein loading in each lane. Results are means ± SE. * p<0.05 vs. respective vehicle (Veh) groups; # p<0.05 vs. respective TGF-β1 alone groups by one way ANOVA.

Figure 2. Representative Western blot analysis demonstrating that A) pretreatment with cGMP does not block TGF-β1-induced phosphorylation of Smad2 or Smad3 and B) Neither cGMP nor TGF-β1 treatment altered total Smad2/3 levels in rat PASMCs. Quiescent PASMCs were stimulated with TGF-β1 (1 ng/ml) for 30 min with or without pretreatment with cGMP (8-Br-cGMP, 0.01 to 1 mM for 30 min). Smad4 levels were not altered by TGF-β1 or cGMP treatment. Cell lysates (25 μg) were size fractionated by SDS-PAGE, and Western blot analysis was performed with selective anti-phospho-Smad2 (pSmad2), anti-phospho-Smad3 (pSmad3), anti-Smad4, or anti-Smad2/3 antibodies. Anti-Smad2/3 antibody can not distinguish Smad2 and Smad3. Veh = vehicle control: PASMCs cultured in 0.1% FBS medium.

Figure 3. Representative fluorescent micrographs show that ANP and cGMP inhibit TGF-β1-stimulated nuclear translocation of A) pSmad2 and B) pSmad3 in rat PASMCs. PASMCs were cultured on slides. After starvation in 0.1% FBS medium for 24 hrs, PASMCs were pretreated with ANP (1 µM), cGMP (8-Br-cGMP, 1 mM) or vehicle for 30 min and then exposed to TGF-β1 (1 ng/ml) for an additional 1 hr. Fixed cells were stained with selective anti-pSmad2 or anti-pSmad3 antibodies and a Texas Red-labeled secondary antibody.

Figure 4. Confocal fluorescent micrographs show that cGMP inhibits TGF-β1-stimulated nuclear translocation of A) pSmad2 and B) pSmad3 in rat PASMCs. PASMCs were cultured on slides. Quiescent PASMCs were pretreated with cGMP (8-Br-cGMP, 1 mM) or vehicle for 30 min and then exposed to TGF-β1 (1 ng/ml) for an additional 1 hr. Fixed cells were stained with selective anti-pSmad2 or anti-pSmad3 antibodies and a Texas Red-labeled secondary antibody. Nuclei were stained with DAPI.

Figure 5. (A & B) Inhibitory effects of ANP (1 µM for 30 min) and cGMP (8-Br-cGMP, I mM for 30 min) on TGF-β1 (1 ng/ml for an additional 30 min)-stimulated nuclear translocation of pSmad2 and pSmad3 in rat PASMCs. Vehicle (Veh) was PASMCs cultured in 0.1% FBS medium for 24 hrs. Fixed cells were incubated with selective anti-pSmad2 or anti-pSmad3 antibody and a Texas Red-labeled secondary antibody. Results are means ± SE. A total of > 500 cells in 10 plates were counted. * p<0.05 vs. respective vehicle groups; # p<0.05 vs. respective TGF-β1 alone group by one way ANOVA. (C & D) Time course of the inhibitory effects of cGMP (8-Br-cGMP, I mM for 30 min) on TGF-β1 (1 ng/ml for additional 5 min to 6 hrs)-stimulated nuclear translocation of pSmad2 and pSmad3 in rat PASMCs. Results are means±SE. A total of > 300 cells in 3 independent experiments were counted.

Figure 6. Effects of ANP, cGMP, TGF-β1, and/or PKG inhibitors on PKG activity in rat PASMCs. PASMCs were pretreated with the PKG inhibitors KT 5823 (1 µM) or Rp-8-Br-cGMP (50 µM) for 15 min prior to ANP (1 µM) or cGMP (8-Br-cGMP, 1 mM), with or without TGF-β1 (1 ng/ml) treatment for an additional 15 min before being harvested for analysis. Vehicle control (lane 1) was growth-arrested PASMCs grown in 0.1% FBS medium. A) Cell lysates were assayed for PKG activity by measuring the incorporation of 32P from [γ-32P] ATP into a selective PKG substrate, Glasstide. B) Cell lysates were assayed for PKG activity by measuring the phosphorylation of vasodilator-stimulated phosphoprotein (VASP). Ser239 is the major PKG phosphorylation site on VASP. Results are means ± SE. n = number of plates. * p<0.05 vs. vehicle control group (lane 1); # p<0.05 vs. respective cGMP or ANP groups by one-way ANOVA.

Figure 7. PKG inhibitors KT5823 and Rp-8-Br-cGMP block the inhibitory effects of ANP and cGMP on TGF-β1 (1 ng/ml)-stimulated nuclear translocation of pSmad3 in rat PASMCs. Vehicle control (lane 1) was growth-arrested PASMCs grown on slides in 0.1% FBS medium for 24 hrs. The order of the treatment was: KT5823 (1 uM), Rp-8-Br-cGMP (50 µM) or vehicle for 15 min; ANP (1 uM), cGMP (8-Br-cGMP, 1 mM) or vehicle for 15 min; TGF-β1 (1 ng/ml) or vehicle for an additional 1 hr. Cells were fixed and stained with selective anti-pSmad3 antibody and a Texas Red-labeled secondary antibody. Results are means±SE. A total of > 300 cells in 3 independent experiments were counted. * p<0.05 vs. vehicle control group (lane 1); # p<0.05 vs. respective groups with TGF-β1 by one-way ANOVA.

Figure 8. Schematic illustration of TGF-β1 signal transduction and postulated mechanisms of inhibition by ANP-cGMP-PKG signaling, i.e. over-phosphorylation of Smad2 and Smad3, resulting in inhibition of nuclear translocation and subsequent interaction with transcriptional regulatory factors and binding to DNA to regulate expression of downstream genes.

ANP INHIBITS TGF-β-INDUCED SMAD SIGNALING AND MYOFIBROBLAST TRANSFORMATION IN MOUSE CARDIAC FIBROBLASTS

by

Peng Li*, Dajun Wang*, Jason Lucas, Suzanne Oparil, Dongqi Xing, Xu Cao, Lea Novak, Matthew B Renfrow, Yiu-Fai Chen

* Equal contribution

Circulation Research. 2008;102:185-192

Copyright 2007

by

Wolters Kluwer Health

Used by permission

Format adapted for dissertation
ABSTRACT

This study tested the hypothesis that activation of atrial natriuretic peptide (ANP)/cGMP/protein kinase G (PKG) signaling inhibits transforming growth factor (TGF)-β1-induced extracellular matrix (ECM) expression in cardiac fibroblasts (CFs) and defined the specific site(s) at which this molecular merging of signaling pathways occurs. Left ventricular (LV) hypertrophy and fibrosis, collagen deposition and myofibroblast (MF) transformation of CFs in response to pressure overload by transverse aortic constriction (TAC) were exaggerated in ANP null mice compared to wild type controls. ANP and cGMP inhibited TGF-β1-induced MF transformation, proliferation, collagen synthesis and plasminogen activator inhibitor 1 (PAI-1) expression in CFs isolated from wild type mice. Following pretreatment with cGMP, TGF-β1 induced phosphorylation of Smad3, but the resultant pSmad3 could not be translocated to the nucleus. pSmad3 that had been phosphorylated with recombinant PKG-1α was analyzed by use of Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) and ion trap tandem mass spectrometry (MS/MS). The analysis revealed phosphorylation of Ser309 and Thr388 residues, sites distinct from the C-terminal Ser423/425 residues that are phosphorylated by TGF-β receptor kinase and are critical for the nuclear translocation and down-stream signaling of pSmad3. These results suggest that phosphorylation of Smad3 by PKG is a potential molecular mechanism by which activation of ANP/cGMP/PKG signaling disrupts TGF-β1-induced nuclear translocation of pSmad3 and downstream events, including MF
transformation, proliferation and expression of ECM molecules in CFs. We postulate that this process contributes to the anti-fibrogenic effects of the natriuretic peptide in heart.

**Key words:** Atrial natriuretic factor, Transforming growth factor, Cardiac fibroblast, Cardiac fibrosis and remodeling, Signal transduction, Protein kinase G, Smad phosphorylation, FT-ICR MS.
INTRODUCTION

Our previous studies have provided shown endogenous atrial natriuretic peptide (ANP) and transforming growth factor (TGF)-β play important counterregulatory roles in pressure overload-induced cardiac hypertrophy, remodeling and fibrosis.1-4 ANP and TGF-β expression are upregulated in heart with pressure overload stress.1 The functional significance of this stress-induced increase in ANP is supported by observations of exaggerated left ventricular hypertrophy (LVH), fibrosis and remodeling in ANP deficient (Nppa/-) mice compared to wild type controls in response to pressure overload.1,2,4 Our recent studies in a novel mouse model that expresses an inducible dominant negative mutation of the TGF-β receptor type II gene (DnTGFβRII), and thus cannot activate the TGF-β/Smad signaling cascade, demonstrated that disruption of TGF-β signaling greatly attenuated the pressure overload-induced MF transformation and interstitial fibrosis in heart, supporting a critical role for TGF-β signaling in the pathogenesis of pressure overload-induced cardiac hypertrophy and remodeling.3

ANP increases intracellular cGMP levels and activates cGMP-dependent protein kinase (PKG), with resultant growth inhibiting and anti-growth/proliferative effects in a variety of cell types, including CFs,5 while, activated TGF-β stimulates cellular differentiation, transformation, proliferation, migration, and ECM expression.6-8 TGF-β signals through membrane bound heteromeric type I (TGFβRI) and type II (TGFβRII) receptor kinases that transduce intracellular signals via phosphorylation and nuclear translocation of receptor-activated
Smad2 and Smad3 proteins, which modulate the transcription of many genes.\textsuperscript{9} Phosphorylation of the C-terminal Ser423/425 of Smad3 by the TGF-\(\beta\) receptor kinase is critical for its nuclear translocation and downstream signaling. The molecular mechanisms of the counterregulatory effects of ANP/cGMP/PKG signaling on activated TGF-\(\beta\)-induced Smad signaling in cardiac cells have not been studied.

The present study tested the hypotheses that TGF-\(\beta\)\textsubscript{1} accelerates myofibroblast (MF) transformation and extracellular matrix (ECM) production in cardiac fibroblasts (CFs) and that activation of the ANP signaling cascade inhibits these processes by interrupting TGF-\(\beta\)\textsubscript{1} signaling. Specifically, we tested whether ANP/cGMP/PKG signaling interrupts specific downstream events in the TGF-\(\beta\)\textsubscript{1} signaling pathway, including phosphorylation and nuclear translocation of Smad2 and Smad3. We demonstrated that, following pretreatment with cGMP, TGF-\(\beta\)\textsubscript{1} induced phosphorylation of Smad2 and Smad3, but the resultant pSmads could not be translocated to the nucleus. We then tested the hypothesis that PKG has the potential to phosphorylate Smad3 at sites different from those required for its TGF-\(\beta\)\textsubscript{1}-induced nuclear translocation, thus disrupting its entry into the nucleus and downstream signaling. Two novel sites of Smad3 phosphorylation were identified (Ser309 and Thr388) by use of high resolution mass spectrometry (MS). These differ from the C-terminal Ser423/425 residues that are substrates for phosphorylation by the TGF-\(\beta\) receptor kinase, suggesting a novel mechanism by which the ANP/cGMP/PKG signaling pathway can inhibit the profibrotic effects of TGF-\(\beta\).
MATERIALS AND METHODS

Animal preparation

Male ANP null (Nppa\(^{-/-}\)) mice\(^1\) and wild-type controls (Nppa\(^{+/+}\)) of the C57BL/6 strain were studied. Mice were fed a standard diet (Harlan-Teklad) and were housed in rooms maintained at constant humidity (60±5%), temperature (24±1\(^\circ\)C), and light cycle (6 AM to 6 PM). All protocols were approved by the IACUC at the UAB and were consistent with the Guide for the Care and Use of Laboratory Animals published by the US NIH (DHEW Publication No. 96-01, revised in 2002).

Surgical procedures

Nppa\(^{-/-}\), 9-10 wks of age, and age-matched wild-type Nppa\(^{+/+}\) mice underwent transverse aortic constriction (TAC) or sham surgery under ketamine/xylazine (8 mg/1.2 mg/100g, i.p.) anaesthesia.\(^1,2,4\) Using this methodology, we have previously demonstrated reproducible pressure gradients across the TAC of 50-65 mmHg.\(^1\)

Effects of TAC on collagen deposition and myofibroblast transformation in hearts of Nppa\(^{+/+}\) and Nppa\(^{-/-}\) mice

One wk after TAC, mice were killed with an overdose of pentobarbital and by cervical dislocation. Heart were dissected and weighed. The left ventricle (LV) was fixed with 4% paraformaldehyde, paraffin embedded and sectioned for morphologic and immunohistochemical examination of collagen deposition and MF transformation. Collagen was assessed using picrosirius red staining and
MFs were identified by α-smooth muscle actin (α-SMA, using clone1A4 anti-α-SMA antibody, Dako) immunochemical staining. Adjacent cross sections (5 μm) from LV were examined to assess colocalization of collagen and α-SMA in or near the same cell types. In a subgroup of wild-type mice, TGF-β1 (total and active) protein levels in LV were measured using an ELISA kit (R&D Systems).

**Cardiac fibroblast preparation**

CFs were isolated from hearts of adult male C57BL/6 mice weighing 18-25 grams. Hearts were excised, rinsed in cold Hank’s balanced salt solution, minced, and digested with collagenase type 4 (100 U/ml) and trypsin (0.6 mg/ml) at 37°C for 30 min. The first digestion was discarded. The collagenase medium from the second digestion containing the CFs was centrifuged for 10 min at 180g, and resuspended in DMEM with 15% fetal bovine serum (FBS). The digestion was repeated until the digestion fluid became clear (5-6 times). Cells were plated in laminin-coated 60 mm dishes (Becton Dickinson) and allowed to attach for 45 min prior to the first media change, which removed weakly adherent cells, including myocytes and endothelial cells. Passage 1 CFs were used for the experiments.

**Effects of cGMP on TGF-β1-induced myofibroblast transformation in cultured mouse CFs**

CFs isolated from wild-type mice were grown in 15% FBS-DMEM to 85% confluence on 18 mm² glass cover slides and then made quiescent by culturing them in 0.1% FBS medium for 48 hrs. Quiescent CFs were pre-treated with cGMP (8-Br-cGMP, a cGMP analog, 10⁻³ M) (Sigma) or vehicle for 30 min, and
then exposed to TGF-β1 (1 ng/ml) (Sigma) or vehicle for an additional 24 hrs. Cells were then fixed with 4% paraformaldehyde and immunostained for α-SMA as a marker of MF transformation and counterstained with hematoxylin for visualization of nuclei. Quantitative analysis for α-SMA positive cell/total cell ratios was carried out by light microscopy with a Qimaging QICam color camera (Qimaging) interfaced with a computer system running MetaMorph software (Universal Imaging Corporation).

**Effects of ANP on cGMP levels, and effects of ANP/cGMP on collagen synthesis, cell proliferation and plasminogen activator inhibitor (PAI)-1 expression in mouse CFs**

Quiescent mouse CFs were treated with ANP (1 µM) (Sigma) for 30 min, then harvested for cGMP measurement using a standard radioimmunoassay kit (Amersham). CFs isolated from adult male Spraque-Dawley rats were isolated and treated similarly and used as controls.

Separate groups of quiescent CFs were pretreated with ANP (1 µM) or cGMP (1 mM) for 30 min prior to exposure to TGF-β1 (5 ng/ml) for an additional 24 hrs and de novo collagen synthesis in CFs was evaluated by measuring [³H]-proline incorporation into cells using the method of Maki et al.¹¹

To test the hypothesis that cGMP inhibits TGF-β1-stimulated cell proliferation, quiescent CFs were pretreated with cGMP (1 mM) for 30 min prior to exposure to TGF-β1 (5 ng/ml). Cell proliferation was measured using a CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega) at 30, 90, and 240 min after TGF-β1 treatment.
The effects of cGMP on TGF-β1-stimulated PAI-1 mRNA expression (a biomarker of TGF-β action in cells) was tested in quiescent CFs treated with TGF-β1 (5 ng/ml) for 24 hrs with or without pretreatment with cGMP (1 mM for 30 min) and/or the PKG inhibitor KT5823 (1 µM, 15 min prior to cGMP) (CalBiochem). Northern blot analysis for PAI-1 and GAPDH (internal control) mRNA levels was performed.1,12

**Effects of ANP/cGMP on TGF-β1-induced nuclear translocation of pSmad3 in mouse CFs**

To test the hypothesis that inhibition of TGF-β1-stimulated MF transformation and ECM expression by ANP/cGMP/PKG signaling is dependent on events downstream from phosphorylation of Smad2 and Smad3, we examined the effects of ANP and cGMP on TGF-β1-stimulated nuclear translocation of pSmad2 and pSmad3 in CFs. Quiescent mouse CFs were pretreated with ANP (1 µM), cGMP (1 mM) or vehicle for 30 min, and then exposed to TGF-β1 (1 ng/ml) for an additional 30 min. Subgroups of CFs were pretreated with the PKG inhibitor KT5823 (1 µM) for 15 min prior to ANP or cGMP. CFs were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X100 in PBS. The fixed CFs were stained with selective anti-phospho-Smad2, anti-phospho-Smad3 or anti-Smad2/3 primary antibodies (1:400X, Cell Signaling Technology) overnight at 4ºC and then with a Texas-conjugated donkey anti-rabbit IgG secondary antibody (1:500X, Jackson ImmunoResearch Lab) for 1 hr at room temperature to assess nuclear translocation of pSmad2 and
Effects of TGF-β1 and cGMP on phosphorylation of Smad proteins

Quiescent CFs were pretreated with cGMP (1 mM) or vehicle for 30 min before addition of TGF-β1 (1 ng/ml) to the medium and incubated for an additional 30 min, then harvested for assessment of pSmad2 and pSmad3 using Western blot analysis.12

We then tested the hypothesis that cGMP/PKG activation can phosphorylate Smad3 protein at sites other than the C-terminal SSXS motif, in which the two end serines (Ser423/425) are phosphorylated by the TGF-β type I receptor kinase.13 A plasmid with a glutathione S transferase (GST)-Smad3 fusion gene was transfected into E. coli and expression of fusion proteins was induced by isopropyl-B-D-thiogalactoside (IPTG). Proteins were harvested with glutathione beads and cleaved with thrombin to yield purified Smad3 as described previously.14 Purified Smad3 (10 µg) was first phosphorylated with a recombinant PKG-1α (1000 U for 20 min, CalBiochem) using γ-32P-ATP or non-radioactive ATP as substrate, then digested with carboxypeptidase Y (a C-terminal peptidase, 1 ng/µl, Sigma) for 1, 30, and 60 min. The reaction mixture was subjected to 10% SDS-PAGE. The gel with 32P-Smad3 was used for radioautography to identify the phosphorylated peptides and the gel with non-radioactive pSmad3 was used for Western blot analysis to identify the Smad3

pSmad3 using confocal fluorescence microscopy with a computerized Zeiss-Axioskop system.
peptides. The Western blot was probed with either a selective anti-N-terminal Smad3 antibody or an anti-C-terminal-Smad3-pSer423/S425 antibody.

**Identification of the PKG phosphorylated sites on Smad3 protein by FT-ICR MS**

Two µg of PKG-1α-phosphorylated Smad3 (n=6) and unphosphorylated (reaction without PKG in the buffer, as a negative control, n=2) were dissolved in 100 µl 50 mM ammonium bicarbonate solution. pSmad3 was first reduced in 10 mM dithiothreitol (DTT) (by adding 5 µl of 200 mM DTT to the reaction mixture) for 30 min, alkylated in 50 mM iodoacetamide (by adding 5 µl of 1 M iodoacetamide to the reaction mixture) for 30 min, and then digested with 0.2 µg (by adding 5 µl of 40 µg/ml enzyme to the reaction mixture) trypsin (Promega) or GluC (Roche) for 24 hr at room temperature. The trypsin- or GluC-digested pSmad3 peptides were then analyzed by use of reversed phase C18 liquid chromatography (RP-C18 LC)-Fourier Transform-ion cyclotron resonance (FT-ICR) mass spectrometry (MS) analysis and ion trap tandem mass spectrometry (MS/MS) as previously described.15,16

**Statistical analysis**

Results are expressed as means±SE. Statistical analyses were carried out using the SigmaStat package (Jandel Scientific Software, San Rafael, CA) on a PC. The primary statistical test was analysis of variance (ANOVA). If ANOVA results were significant, a post-hoc comparison among groups was performed.
with the Newman-Keuls test. Differences in mean values were reported as significant if the P value was < 0.05.

RESULTS

Myofibroblast transformation and collagen deposition are increased in hearts of Nppa-/- mice compared to Nppa+/+ mice subjected to TAC

In response to TAC, Nppa-/- mice developed significant cardiac hypertrophy [LV weight (adjusted by body weight): 209±6 mg in TAC-Nppa-/- mice (n=10) vs. 124±3 mg in Sham-Nppa-/- mice (n=8)], with greatly increased numbers of MFs [α-SMA antibody stained cells that are not vascular smooth muscle cells (VSMCs) or cardiomyocytes (CMs)] and more robust collagen protein expression (picrosirius stain) compared to wild-type (Nppa+/+) mice [LV weight (adjusted by body weight): 113±3 mg in TAC-Nppa+/+ mice (n=9) vs. 99±2 mg in Sham-Nppa+/+ mice (n=9)] (Figure 1). MFs were colocalized with collagen in the hypertrophic LV in both genotypes (Figures 1A vs. 1B and 1C vs. 1D). No MFs (data not shown) or significant collagen deposition was observed in sham operated Nppa-/- or Nppa+/+ mice (Figure 1E). Higher active TGF-β1 protein levels were observed in LV of wild-type TAC mice than in sham operated controls (Figure 1F).

Expression of α-SMA was also induced in some CMs in LV of TAC mice (data not shown). The α-SMA in CMs was not colocalized with collagen, suggesting that these cells are likely not synthesizing ECM.

These results suggest that ANP is a negative modulator of MF
transformation in response to pressure overload stress and that the exaggerated cardiac hypertrophy/remodeling observed in \textit{Nppa-/-} mice in response to TAC is related to increased MF transformation and ECM deposition.

**Cyclic-GMP inhibits TGF-\(\beta\)-1-induced myofibroblast transformation of mouse CFs in vitro**

MFs in vehicle treated cultures (0.1\% FBS medium) were sparse and characterized by disorganized intracellular \(\alpha\)-SMA (\% \(\alpha\)-SMA positive cells=27\pm5\%, \(n=4\) plates/group) (Figures 2A and 2E), while most (95\pm3\%, \(n=4\)) cells in TGF-\(\beta\)1 treated cultures were \(\alpha\)-SMA positive and contained well organized \(\alpha\)-SMA filaments, indicating nearly complete transformation of CFs to MFs (Figure 2B). cGMP completely inhibited TGF-\(\beta\)1-induced MF transformation (\% \(\alpha\)-SMA positive cells=21\pm5\%, \(n=4\)), but did not change basal (\% \(\alpha\)-SMA positive cells=23\pm5\%, \(n=4\)) MF numbers (Figures 2C, 2D and 2E). Together with our in vivo data, these results support the hypothesis that ANP (through cGMP) inhibits TGF-\(\beta\)-induced MF transformation and ECM deposition.

**ANP increases cGMP expression, and ANP and/or cGMP inhibit TGF-\(\beta\)-stimulated collagen synthesis, cell proliferation and PAI-1 mRNA expression in CFs in vitro**

Exposure to ANP (10\(^{-7}\) M) for 30 min increased cGMP levels in both mouse and rat CFs (Figure 3A). TGF-\(\beta\) increased collagen synthesis (\(^{3}[^{\text{H}}]\)-proline incorporation into CFs) (Figure 3B) and cell proliferation (total dehydrogenase activity) (Figure 3C), and pretreatment with ANP or cGMP decreased basal
collagen synthesis and inhibited TGF-β-induced collagen synthesis and proliferation of mouse CFs.

Pretreatment with cGMP decreased baseline levels of PAI-1 and significantly attenuated TGF-β1-stimulated PAI-1 mRNA expression (Figure 3D). Pretreatment with the PKG inhibitor KT5823 blocked the inhibitory effects of cGMP on TGF-β1-stimulated PAI-1 mRNA expression in CFs, suggesting that cGMP was acting through activation of PKG. These data support the hypothesis that ANP/cGMP/PKG signaling has anti-fibrogenic effects that antagonize TGF-β-induced stimulation of ECM expression in CFs.

**ANP and cGMP inhibit TGF-β-induced nuclear translocation of phosphorylated-Smad3 in mouse CFs**

In vehicle treated cells, immunostaining of pSmad3 (Figure 4A and 4E) and pSmad2 (data not shown) was weak and distributed evenly in cytoplasm and nucleus, suggesting that pSmad3 and pSmad2 levels were low and without significant nuclear translocation. TGF-β1 (1 ng/ml for 30 min) treatment significantly stimulated nuclear translocation of pSmad3, indicated by strong pSmad3 staining in the nucleus (Figure 4B and 4E). We did not observe significant pSmad2 nuclear translocation after 30 min of TGF-β1 treatment in mouse CFs (data not shown). Nearly 100% of TGF-β1 treated CFs had accumulated pSmad3 in their nuclei, and > 30% of CFs had double nuclei, indicating cell proliferation. Pretreatment with ANP (1 µM for 30 min) or cGMP (1 mM for 30 min) inhibited TGF-β1-induced pSmad3 translocation, with substantial levels of Smad3 staining remaining in the cytoplasm in most cells (Figures 4C,
To test whether activation of PKG mediates the inhibitory effects of ANP and cGMP on TGF-β1-induced nuclear translocation of pSmad3, pSmad3 nuclear translocation was measured in the presence of the PKG inhibitor KT5823. Pretreatment with KT5823 blocked the inhibitory effects of ANP and cGMP on TGF-β1-stimulated pSmad3 nuclear translocation (Figure 4F).

Cyclic-GMP does not inhibit TGF-β1 induced pSmad3 and pSmad2 phosphorylation

Western blot analysis demonstrated that TGF-β1 treatment significantly increased pSmad3 and pSmad2 levels in CFs and that pretreatment with cGMP did not inhibit this process (Figure 5A). Neither cGMP nor TGF-β altered total Smad2/3 levels in these cells. Thus, disruption of Smad3 and Smad2 phosphorylation does not account for the inhibitory effects of ANP and cGMP on TGF-β1-induced ECM expression in CFs.

PKG phosphorylates Smad3 protein at sites other than the C-terminal residues

As an initial test of the hypothesis that PKG can phosphorylate Smad3 protein at novel sites other than the C-terminal Ser423/425 residues that are phosphorylated by the TGF-β type I receptor kinase, PKG-phosphorylated Smad3 was digested with carboxypeptidase Y and size fractionated by SDS-PAGE. Autoradiographic analysis and Western blots of pSmad3 fragments probed with either a selective anti-N-terminal Smad3 antibody or an anti-C-terminal-Smad3-pSer423/S425 antibody indicated that PKG did phosphorylate
Smad3 when the C-terminal residues were removed by a C-terminal peptidase (Figure 5B), supporting the hypothesis that PKG can phosphorylate Smad3 at novel sites other than its C-terminal residues.

RP-C18-LC FT-ICR-MS/MS analysis of trypsin- or Gluc-digested pSmad3 that was phosphorylated by PKG confirmed that Ser309 and Thr388 on pSmad3 was phosphorylated by PKG (Figure 6). Figure 6B shows the FT-ICR MS spectrum of a doubly charged ion species at m/z 822.3542 corresponding to the mass of the pSmad3 tryptic peptide L296-S309 plus the addition of a phosphate group (theoretical, 1643.7063, 3.18 ppm mass error). Figure 6C shows the LTQ MS/MS product ion spectrum of the same ion after fragmentation with the dominant fragment corresponding to the neutral loss of 80 Da, confirming the phosphorylated pSmad3 peptide. Phosphorylation of Thr388 was confirmed in similar fashion (data not shown).
DISCUSSION

This study yields novel insights into precise sites of “molecular merging” of pro- and anti-fibrogenic pathways in heart and characterizes CFs as the target cell in which pro- and anti-fibrogenic signaling cascades converge and regulate responses of the heart to hemodynamic stress. We demonstrate crosstalk between ANP and TGF-β signaling pathways in CFs in vitro such that ANP and cGMP, through a PKG dependent mechanism, block induction of ECM and PAI-1 expression by TGF-β1. Our observation that cGMP inhibits TGF-β1-induced nuclear translocation of pSmad2 and pSmad3 in CFs defines for the first time a precise molecular mechanism by which ANP/cGMP/PKG signaling interferes with downstream signaling from TGF-β and thus protects against cardiac remodeling/fibrosis and failure in response to hemodynamic stress. These findings suggest the intriguing hypothesis that ANP signaling results in phosphorylation of Smad proteins on sites other than their C-terminal residues, thus blocking their nuclear translocation and binding to TGF-β-Smad responsive elements in the promoter regions of ECM genes.

TGF-β1, the major isoform in heart, is produced in CFs and CMs under stressful conditions and stimulates CF transformation (to MFs) and proliferation, as well as ECM production in response to hypertrophic stimuli. Thus, it plays a major role in cardiac remodeling under stress conditions.

Activated TGF-β ligands bind to a heteromeric complex of type II (TGFβRII) and type I (TGFβRI) receptors that transduce intracellular signals via phosphorylation of TGFβRI-associated Smad2 and Smad3. Phosphorylation
of TGFβRI-associated Smad2 and Smad3 and nuclear translocation of pSmad2 and pSmad3 are critical steps in TGF-β signaling.\textsuperscript{14,18} Smad2 (467 a.a.) and Smad3 (425 a.a.) contain predominantly serine, with some threonine residues, in the C-terminal, linker and MH1 regions that are accessible for phosphorylation. Upon ligand binding, phosphorylation by TGFβR1 kinase of the two most C-terminal serine residues drives the activation of Smad2 and Smad3 is required for nuclear translocation and subsequent binding of pSmad2 and pSmad3 to nuclear transcriptional factors and DNA that regulate the transcriptional expression of downstream genes.\textsuperscript{14,18}

The interplay between Smads and other signaling pathways in the cytoplasm and nucleus is a critical mechanism by which the activities and expression of Smad proteins are modulated, and is responsible for the diverse effects of the TGF-β family of proteins.\textsuperscript{14} Mice overexpressing TGF-β have cardiac hypertrophy and interstitial fibrosis,\textsuperscript{8} and studies in rat and human CFs have shown that B-type Natriuretic peptide (BNP) and nitric oxide (NO) attenuate expression of TGF-β through activation of cGMP. However, the signaling cascades involved remain to be elucidated.\textsuperscript{8,19}

Both inhibition- and over-phosphorylation of Smad2 or Smad3 have been reported to disrupt their heterodimerization with Smad4 and nuclear translocation, resulting in repression of transcriptional activation of TGF-β responsive promoters.\textsuperscript{20,21} Thus, phosphorylation not only activates Smad proteins but also modulates their activity. This provides a potential mechanism for integration of the Smad pathway with ANP/cGMP/PKG signaling pathway that
modulates TGF-β signal transduction. The current study demonstrates that the
PKG acts as a stronger protein kinase than TGFβRII and phosphorylates
additional serine or threonine residues on Smad3 (and/or Smad2), thus
disrupting their nuclear translocation, resulting in repression of transcriptional
activation of TGF-β response promoters, e.g. on collagen and PAI-1 genes. This
represents a novel “molecular merging” mechanism by which ANP/cGMP/PKG
signaling intercepts the TGF-β signaling cascade and may contribute to the anti-
fibrogenic effects of ANP in the stressed heart.

We recognize that over-phosphorylation of Smads is not the only cellular
function of PKG activation. PKG has diverse intracellular actions, including
integrin signal transduction, modulation of Ca²⁺ release and uptake into SR,
alteration of membrane K⁺ fluxes, and nuclear protein phosphorylation and
translocation. An alternative (to over-phosphorylation) explanation for the
observation that ANP signaling prevents nuclear translocation of Smads is that
cGMP and PKG may alter the affinity of Smads for cytoplasmic anchoring
molecules or nuclear export proteins. Subcellular localization of Smads has been
shown to be controlled by interaction with these cytoplasmic and nuclear
retention factors. The precise molecular basis for retention of pSmads in the
cytosol following ANP or cGMP treatment remains to be identified and is a topic
for current investigation in our laboratory.

Several recent studies have demonstrated that various cytoplasmic protein
kinases and cyclic nucleotides participate in regulating responses to TGF-β. ERK MAP kinase inhibits TGF-β signaling via inhibition of nuclear accumulation
of Smad2 in normal epithelial cells. Protein kinase C (PKC) directly phosphorylates Smad3 and abrogates the ability of Smad3 to bind directly to DNA, leading to impairment of transcriptional responses dependent on the direct binding of Smad3 to DNA. Activation of Ca++-calmodulin-dependent protein kinase II prevents Smad2/4 heterodimerization and nuclear translocation and concomitant transcriptional responses in HEK-293 human kidney fibroblasts. Further, intracellular cAMP-elevating agents such as prostaglandin E2 (PGE2), and the adenylate cyclase activator forskolin, inhibit TGF-β-induced Smad3/4-dependent gene expression via a cAMP-dependent protein kinase A (PKA)-dependent mechanism in human keratinocytes. The current study shows that ANP and cGMP inhibit TGF-β-induced nuclear translocation of pSmad2 and pSmad3 in CFs, defining a new role for cyclic mononucleotide phosphate second messenger in regulating pro-fibrogenic responses to TGF-β. Pretreatment with the PKG inhibitors KT5823 prevented the inhibitory effects of ANP and cGMP on TGF-β-stimulated nuclear translocation of pSmad3, supporting the hypothesis that these effects are mediated through activation of PKG.

Our previous in vivo studies have validated the importance of ANP and TGF-β and ANP as opposing influences in the pathogenesis of pressure overload-induced cardiac hypertrophy, fibrosis and remodeling. We have shown that Nppa−/− mice develop cardiac enlargement and remodeling in response to pressure overload stress, compared to Nppa+/+ mice. Similarly, other investigators have shown that Npr1−/− mice carrying targeted-disruption of Npr1 gene (encoding for NPRA receptor) exhibit the same phenotype of cardiac
hypertrophy and fibrosis as Nppa-/- mice, associated are with reduced guanyly cyclase activity and cGMP levels and increased expression of angiotensin-converting enzyme (ACE) and angiotensin II, as well as proinflammatory cytokines such as TNF-α, IL-6 and TGF-β1 in heart, suggesting that disruption of ANP/NPRA/cGMP signaling leads to increases in cardiac hypertrophic stimuli.27,28 We have shown that the excess cardiac enlargement and remodeling in Nppa-/- mice are a consequence of increased MF transformation and proliferation, and deposition of ECM components, rather than excess CM hypertrophy.1 In contrast, using a novel DnTGFβRII mouse model, these processes are markedly attenuated by disruption of TGF-β signaling in heart.3-4 Taken together, these data support the hypothesis that endogenous ANP and TGF-β play important counterregulatory roles in regulating MF transformation and proliferation, ECM production and cardiac remodeling in response to pressure overload stress. An imbalance in the normal relationships between the anti-growth/anti-fibrogenic effects of ANP and mitogenic/pro-fibrogenic of TGF-β results in pressure overload-induced cardiac fibrosis and remodeling.
ACKNOWLEDGMENTS

This work was supported in part by NIH Grants HL-080017, HL-044195, HL-075614, HL-07457, HL-64614, HL-075211, CA-101955, DK60913, and an AHA-0455197B GIA.

CONFLICT OF INTEREST DISCLOSURES

None
REFERENCES


28. Vellaichamy E, Zhao D, Somanna N, Pandey KN. Disruption of guanylyl cyclase/natriuretic peptide receptor-A gene upregulates AT1 receptor signaling in null mutant mice: Role in cardiac hypertrophy. Physiol Genomics. 2007 Jun 12; [Epub ahead of print]
Figure 1. (A-D) Representative micrographs (100X) of collagen (A and C, picrosirius-red staining) and α-SMA positive myofibroblasts (B and D) in LV of male Nppa-/– and Nppa+/+ mice 1 wk post TAC. Arrows indicate myofibroblasts. (E) Interstitial collagen volume calculated in picrosirius-red-stained cross-sections of the LV below the mitral valve in control or TAC (1-wk) Nppa-/– and Nppa+/+ mice. (F) Effects of 1-wk of TAC on total and active TGF-β1 protein levels in LV of Nppa+/+ mice. Results are means±SE, (n)= numbers of mice. * p<0.05 compared with respective Nppa+/+ groups; # p<0.05 compared with respective sham-operated controls by two way ANOVA.

Figure 2. (A-D) Representative micrographs (400X) and (E) bar graphs of means±SE of α-SMA-stained cultured mouse CFs treated with TGF-β1 (1 ng/ml for 24 hrs) and/or cGMP (8-Br-cGMP, a cGMP analog, 1 mM for 25 hrs or 1 hr prior to TGF-β1). Slides were counterstained with hematoxylin to show nuclei. A total of >500 cells were counted in four slides per group in two experiments. * p<0.05 compared with vehicle control group; # p<0.05 compared with TGF-β1 alone group.

Figure 3. (A) Effects of ANP (0.1 µM for 30 min) on intracellular cGMP levels in mouse and rat CFs. (B) to (D) Effects of ANP (1 µM) or cGMP (1 mM) on TGF-β1 (5 ng/ml) stimulated collagen synthesis, cell proliferation and PAI-1 mRNA expression in mouse CFs. In (B) and (D), quiescent CFs were pretreated with ANP or cGMP for 30 min prior to TGF-β1 for an additional 24 hrs before being harvested. In (C), proliferation of CFs (n=24 wells/group) was measured using the Non-Radioactive Cell Proliferation Assay (Promega) at 30, 90, and 240 min after TGF-β1 treatment. MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxy-methoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, a dehydrogenase substrate) and PMS (phenazine-methosulfate, an electron coupling reagent) were added to medium at 30 min post TGF-β1 treatment for measurement of formazan formation as an index of cell proliferation. In (D), subgroups of CFs were pretreated with KT5823 (1 µM) for 15 min prior to cGMP or TGF-β1; Northern analysis was carried out with 15 µg of total RNA and PAI-1 mRNA data were normalized to GAPDH mRNA. Results are means±SE, n=dishes/group. * p<0.05 compared with vehicle control group; # p<0.05 compared with TGF-β1 alone group.

Figure 4. (A-D) Representative micrographs show that cGMP inhibits TGF-β1-stimulated nuclear translocation of pSmad3 in mouse CFs. CFs were cultured on cover slides in 0.1% FBS-DMEM, (B1-B3) and (C1-C3) CFs were treated with TGF-β1 (1 ng/ml) for 30 min and cGMP (1 mM) for 60 min, respectively. (D1-D3) CFs were treated with cGMP (1 mM) for 30 min prior to TGF-β1 (1 ng/ml) for an additional 30 min. Fixed CFs were stained with an anti-pSmad3 antibody and a Texas Red-labeled secondary antibody. Arrows indicate representative CFs with accumulation of pSmad3 in cells with double nuclei. A1-D1, taken with a fluorescent microscope (100X); A2-D2, taken with a confocal fluorescence microscope (400X); A3-D3, nuclei of A2-D2 were stained with DAPI. (E) Relative intensity of pSmad3 staining measured in nucleus and cytoplasm of CFs treated with vehicle, cGMP (1 mM) and/or TGF-β1 (1 ng/ml) as in A2-D2. Results are means±SE, n= number of cells measured; a total of >20 fields (10 in nucleus and 10 in cytoplasm) in each cell were scanned and averaged. (F) Quiescent CFs were pretreated with ANP (1 µM) or cGMP (1 mM) prior to TGF-β1 (1 ng/ml) for an additional 30 min before stained for pSmad3. Subgroups of CFs were pretreated with KT5823 (1 µM) for 15 min prior to ANP or cGMP. Y-axis scale represents percent of total cells with pSmad3 nuclear translocation. Results are means±SE, n=number of slides/group; a total of > 200 cells were counted per group. * p<0.05 compared with vehicle control group; # p<0.05 compared with TGF-β1 alone group.

**A. cGMP did not block TGF-β1-induced pSmad phosphorylation**

![Western blots and averaged bar graphs of pSmad3 demonstrating that cGMP does not block TGF-β1-induced phosphorylation of Smad3 and Smad2 in mouse CFs. Quiescent CFs were stimulated with TGF-β1 (1 ng/ml) for 30 min with or without pretreatment with cGMP (1 mM for 30 min). Cell lysates (25 μg) were size fractionated by SDS-PAGE, and Western analysis was performed with selective anti-pSmad3, anti-Smad2, and β-actin antibodies. β-actin protein levels were measured to show protein loading. Veh=vehicle control; CFs cultured in 0.1% FBS medium. Results are means±SE, n=samples per group. * p<0.05 compared with vehicle control group; # p<0.05 compared with TGF-β1 alone group.](image1)

**B. PKG-phosphorylated Smad3 digested by carboxypeptidaseY**

![PKG-phosphorylated Smad3 digested by carboxypeptidaseY](image2)

Figure 5. (A) Representative Western blots and averaged bar graphs of pSmad3 demonstrating that cGMP does not block TGF-β1-induced phosphorylation of Smad3 and Smad2 in mouse CFs. Quiescent CFs were stimulated with TGF-β1 (1 ng/ml) for 30 min with or without pretreatment with cGMP (1 mM for 30 min). Cell lysates (25 μg) were size fractionated by SDS-PAGE, and Western analysis was performed with selective anti-pSmad3, anti-Smad2, and β-actin antibodies. β-actin protein levels were measured to show protein loading. Veh=vehicle control; CFs cultured in 0.1% FBS medium. Results are means±SE, n=samples per group. * p<0.05 compared with vehicle control group; # p<0.05 compared with TGF-β1 alone group.

(B) Radioautographs and Western blots of purified Smad3 protein that was phosphorylated with PKG (20 min) and then digested with carboxypeptidase Y for 1, 30, and 60 min. Western analysis of carboxypeptidase Y-digested pSmad3 was performed with selective anti-Smad3-N-terminal or anti-Smad3-C-terminal antibodies.

A. **Phosphorylated Sites on Smad3 by PKG**

| 1 | MSSILPFTPP IVKRLGK W K GEQNGQEEKW CEKAVKSLVK KLUKTGQLDE |
| 51 | LEKAITTTQTV NTKCITIPRS LDGRLQVSHR KGLPHV Y CR LWRWPDLHSH |
| 101 | HELRAMELCE FAFNMK KD EV CVNPHYQRV ETPVLPVLV PRHTEIPAEF |
| 151 | PPLDDYSHSI PENTNFPA GI EPQSNIPETP PPGYLSEDGE TSDHQMNHSM |
| 201 | DAGSPNLSPN P M SPAHNI NL D LQPVYCEPA FWC S SYYEL NQRYGETFA |
| 251 | SQPSMTVDGF TDPSNSERFC LGLLLSNVRN AAVELTRRI GRGVRLYYIG |
| 301 | GEVFACLSD SAI FQSPNC NOQRYGWHPAT VCKIPPCGONL KIFNQEEFAA |
| 351 | LLACSVNQGF EAVQVLTRMC TIRMSFVKGW GAEYRRQVT STPCWIELHL |
| 401 | NGPLQMLVKV L TQMGSPSIR CSVS |

Figure 6. MS/MS analysis of Smad3 that has been phosphorylated with PKG (n=6 for either trypsin- or Gluc-digestion during the analysis). Solid underlined portion=MH2 domain; and dashed underlined portion=MH2 domain. More than 99% of Smad3 peptide sequence (except V424 and S425) was detected by MS/MS analysis. Asterisks mark the phosphorylation sites (Ser309 and Thr388) identified by MS/MS in both trypsin- and Gluc-digested pSmad3 fragments. These two sites were not phosphorylated in negative controls (unphosphorylated Smad3, n=2).

(B) LTQ-FT-ICR spectrum of single ion isolated from a LC-MS of trypsin digested Smad3 that had been incubated with PKG. The ion species matches the theoretical mass of the Smad3 tryptic peptide + HPO3. (C) LTQ tandem mass spectrum of the same ion shows a neutral loss of 80 Da corresponding to the loss of HPO3 that is characteristic of phosphopeptides, confirming phosphorylation of Smad3.

DISCUSSION

INHIBITION OF TRANSFORMING GROWTH FACTOR (TGF)-β SIGNALING INDUCES LEFT VENTRICULAR DILATION AND DYSFUNCTION IN THE PRESSURE OVERLOADED HEART

IN VIVO

Structural remodeling of the myocardial ECM, resulting in interstitial and fibrosis, is the main determinant of myocardial dysfunction and, ultimately, heart failure in our animal models. In our first paper, we have utilized a novel DnTGFBRII mouse model of inducible inhibition of the TGF-β/Smad signaling cascade, originally designed to study TGF-β signaling in bone (51), to define the contribution of TGF-β signaling to the phenotype of cardiac remodeling and fibrosis in response to pressure overload. The DnTGFβRII mouse model offers important advantages for studying the contribution of TGF-β signaling to pressure overload-induced cardiac hypertrophy and remodeling. TGF-β signaling is essential for epithelial-mesenchymal transformation (EMT) during embryonic development of the heart valves, and homozygous deletion of the TGF-β gene leads to embryonic lethality, which has prevented the successful development of a TGF-β knock-out model (29). If the mice do survive until birth, they die within two weeks of wasting syndrome or overwhelming inflammatory responses. The mutation in the DnTGFβRII mouse can be induced in the mature animal, thus does not disrupt critical TGF-β signaling pathways in the developing heart, while
providing the ability to selectively inhibit the downstream signaling of TGF-β at the receptor level later in life. Also, there is no need to repeatedly inject agents into the mice or monitor crests and troughs of drug levels. Our transgenic model works on the genomic level, rendering it superior to models that require pharmacologic interventions.

The major findings of this study are that under pressure overload stress, inhibition of TGF-β signaling results in dramatic reductions in non-myocyte proliferation and collagen content and subsequent development of LV dilation and systolic dysfunction in DnTGFβRII mice. It is well established that under stress the non-myocyte cells in the LV produce ECM proteins to ‘control’ the amount of remodeling in the overloaded ventricle (5, 7, 34). These proliferating interstitial cells have been characterized as cardiac myofibroblasts by α-smooth muscle actin staining in our previous study (34). Our current findings are consistent with our previous observations that TGF-β stimulates proliferation and myofibroblast transformation of isolated mouse cardiac fibroblasts (34) and that non-myocyte proliferation and ECM deposition account for pressure overload-induced LV enlargement in mice subjected to TAC (59). There was minimal apoptotic staining (TUNEL stain) of either fibroblasts or myocytes in LV of any experimental group after seven days of pressure overload, indicating that apoptosis does not play a major role in the LV remodeling observed in these animals.

Inhibition of the TGFβ/Smad signaling pathway attenuated the deposition of collagen in our pressure overload model. In contrast to the other 3 treatment
groups, there was no evidence of pressure overload-related increases in LV collagen levels in DnTGFβRII+Zn++ hearts at any time point post TAC. By 120 days of pressure overload, there was evidence of exaggerated LV dilation and systolic dysfunction, as indicated by increased LV ESV and LVESD, LVEDV and LVEDD, and decreased FS and EF by echocardiographic examination in DnTGFβRII+Zn++ mice. Taken together, these data suggest that interstitial collagen maybe required to maintain ventricular structure under chronic pressure overload stress. This study adds to a growing body of knowledge concerning the role of TGF-β signaling in the pathogenesis of cardiac remodeling/fibrosis in response to various forms of stress in mouse models.

ANP SIGNALING INHIBITS TGF-B INDUCED SMAD2 AND SMAD3 NUCLEAR TRANSLOCATION AND EXTRACELLULAR MATRIX EXPRESSION IN RAT PULMONARY ARTERIAL SMOOTH MUSCLE CELLS

IN VIVO/IN VITRO

Chronic pulmonary hypertension in humans is a debilitating syndrome characterized by increased and sustained pulmonary arterial pressure with concomitant increases in pulmonary vascular remodeling/fibrosis/resistance, frequently leading to respiratory and cardiac failure (4, 9). It has been shown that TGF-β is an important player in hypoxic pulmonary vascular remodeling, demonstrated by significant increases in TGF-β1 mRNA expression in pulmonary arterial walls after exposing rats to 14 days of hypoxia, and increasing expression of α-smooth muscle actin in intra-acinar pulmonary arteries with increased hypoxic times (22). In the present study, we demonstrated for the first time that
ANP has an anti-fibrogenic effect on PASMCs treated with TGF-β1. ANP and cGMP suppressed TGF-β-stimulated ECM gene expression by interfering with Smad signaling through a PKG-dependent mechanism.

The most significant finding of this study is that ANP and cGMP inhibit TGF-β1-induced nuclear translocation of pSmad2 and pSmad3, but not the TGF-β1-induced phosphorylation of Smad2 and Smad3 in PASMCs, thus defining a novel molecular mechanism by which ANP signaling intercepts the TGF-β signaling pathway and blocks TGF-β-induced ECM expression.

TGF-β is an important mediator of pulmonary morphogenesis and of the pathogenesis of pulmonary vascular remodeling (2). Increases in the local abundance of active TGF-β1 promote vascular wall remodeling, arterial lesion growth, and vascular cell differentiation (46). Small amounts of TGF-β are present in a latent, inactive form in the normal adult lung. Expression of TGF-β is increased, as well as activation, in pathological conditions, including hypoxia-induced pulmonary hypertension and vascular remodeling (3). TGF-β1 is a mediator of fibrotic tissue remodeling and is overexpressed several animal models of pulmonary hypertension (13, 45).

In this report we demonstrate increased expression of periostin (PN) in the lung, specifically in PASMCs, in response to TGF-β treatment. This finding, coupled with our previous observation of increased PN expression in lung of mice adapted to hypoxia (10), suggests involvement of this ECM molecule in hypoxia-induced pulmonary vascular remodeling. Our finding that ANP and
cGMP inhibit TGF-β1-induced expression of the ECM molecules PN and osteopontin (OPN), as well as PAI-1, a TGF-β-Smad target gene in PASMCs, indicates that there is a functionally significant interaction between ANP and TGF-β signaling that may play an important role in modulating hypoxia-induced pulmonary vascular remodeling.

**ANP INHIBITS TGF-β-INDUCED SMAD SIGNALING AND MYOFIBROBLAST TRANSFORMATION IN MOUSE CARDIAC FIBROBLASTS IN VITRO**

TGF-β promotes fibrotic tissue remodeling by increasing the production of ECM proteins in the heart, and ANP plays a counterregulatory role to counteract these events. This study characterizes CFs as the target cell in which pro- and anti-fibrogenic signaling cascades converge and regulate responses of the heart to hemodynamic stress. We demonstrate crosstalk between ANP and TGF-β signaling pathways in CFs in vitro such that ANP and cGMP, through a PKG dependent mechanism, block induction of ECM expression by TGF-β1. Our observation that cGMP inhibits TGF-β1-induced nuclear translocation of pSmad2 and pSmad3 in CFs defines for the first time a precise molecular mechanism by which ANP/cGMP/PKG signaling interferes with downstream signaling from TGF-β and thus protects against cardiac remodeling/fibrosis and failure in response to hemodynamic stress. Specifically we have shown that ANP signaling results in phosphorylation of Smad proteins on sites other than their C-terminal residues, thus blocking their nuclear translocation and binding to TGF-β-Smad responsive elements in the promoter regions of ECM genes.
In the TGF-β/Smad signaling pathway, active TGF-β binds to the transmembrane receptor-TGFβRII, which phosphorylates the TGFβRI. The TGFβRI then phosphorylates the Smad2 and/or Smad3 proteins which bind to Smad4 and translocate to the nucleus to promote gene transcription. The phosphorylation of the Smad proteins by the TGFβRI occurs on the C-terminal end of the protein. The current study demonstrates that the interaction of ANP/cGMP/PKG with the TGF-β pathway involves prevention of pSmad3 translocation to the nucleus, but not phosphorylation of Smad3. We show that PKG acts as a stronger protein kinase than TGFβRI in phosphorylating the Smad proteins. We confirm that PKG phosphorylates the Smad protein at sites other than the C-terminal end of the protein. Once the Smad protein has been “overphosphorylated” it probably changes its confirmation and therefore cannot translocate thru the nuclear pores into the nucleus, thus resulting in repression of transcriptional activation of TGF-β responsive promoters, e.g. on collagen and PAI-1 genes. This represents a novel mechanism by which ANP/cGMP/PKG signaling intercepts the TGF-β signaling cascade and may contribute to the anti-fibrogenic effects of ANP in the stressed heart.

Both inhibition- and over-phosphorylation of Smad2 or Smad3 have been reported to disrupt their heterodimerization with Smad4 and nuclear translocation, resulting in repression of transcriptional activation of TGF-β responsive promoters (1, 61). Thus, phosphorylation not only activates Smad proteins but also modulates their activity. This provides a potential mechanism for integration of the Smad pathway with the ANP/cGMP/PKG signaling pathway.
that modulates TGF-β signal transduction. These studies delineate a direct interaction of TGF-β and ANP which may contribute to innovative therapeutic approaches to this important cause of morbidity and mortality in the American population.

**FUTURE DIRECTIONS**

Current unpublished experiments involve studying the crosstalk between TGF-β and Angiotensin II (AngII) signaling in both CFs and PASMCs in regulating ECM production. MAPK pathways activated by G-protein coupled receptor (GPCR) growth factors (e.g. ANGII) play an important role in mediating cell (including CFs) growth and proliferation (12). We are testing which of the three major branches of the MAPK pathway in heart; the Ras-Raf1-MKK1/2-ERK1/2, the Ras-MKK4/7-JNK (c-Jun N-terminal kinase), and the MKK3/6-p38 pathways, is involved in modulating TGF-β signaling (50). Specifically, we are elucidating the exact mechanisms of interaction of Ang II induced Smad3 phosphorylation. (Figure 1 and 2) Our investigation to this point suggests that p38 and JNK have direct involvement in Ang II induced Smad3 activation, with p38 being the more dominant signaling protein. In contrast, ERK 1/2 does not appear to be involved in Ang II induced Smad3 activation in CFs or PASMCs. The intracellular signaling map has been summarized (Figure 3).
1. Effect of ERK1/2 on Ang II-induced Smad3 activation in mice cardiac fibroblasts

Isolated mouse CFs were pre-treated for 15 minutes with specific MAPK inhibitor, followed by treatment with 200nM Ang II for 5 minutes, then protein was harvested. (1) ERK1/2 pathway is not involved in AngII induced Smad3 activation. (2) p38 pathway is directly involved in AngII Smad3 activation. (3) JNK pathway may be involved in AngII induced Smad3 activation. (4) PI3K pathway is not involved in AngII Smad3 activation. Primary antibody: anti-pSmad3 (Cell Signaling). Data was quantitated using Image J software. n=5-9. p<0.05. Internal control: GAPDH.

Figure 1. Representative Western blots and averaged bar graphs for MAPK proteins on Ang II induced Smad3 activation in mouse cardiac fibroblasts. Isolated mouse CFs were pre-treated for 15 minutes with specific MAPK inhibitor, followed by treatment with 200nM Ang II for 5 minutes, then protein was harvested. (1) ERK1/2 pathway is not involved in AngII induced Smad3 activation. (2) p38 pathway is directly involved in AngII Smad3 activation. (3) JNK pathway may be involved in AngII induced Smad3 activation. (4) PI3K pathway is not involved in AngII Smad3 activation. Primary antibody: anti-pSmad3 (Cell Signaling). Data was quantitated using Image J software. n=5-9. p<0.05. Internal control: GAPDH.
Figure 2. Representative Western blots and averaged bar graphs for MAPK proteins on Ang II induced Smad3 activation in rat PASMCs. Isolated rat PASMCs were pre-treated for 15 minutes with specific MAPK inhibitor, followed by treatment with 200nM All for 5 minutes, then protein was harvested. (1) ERK1/2 pathway is not involved in AngII induced Smad3 activation. (2) p38 pathway is directly involved in AngII Smad3 activation. (3) JNK pathway may be involved in AngII induced Smad3 activation. (4) PI3K pathway is not involved in AngII Smad3 activation. Primary antibody: anti-pSmad3 (Cell Signaling). Data was quantitated using Image J software. n=5-9. p<0.05. Internal control: GAPDH.
Figure 3. Schematic illustration of TGFB/Smad and Ang II signaling.
GENERAL LIST OF REFERENCES


33. Li P, Oparil S, Novak L, Cao X, Shi W, Lucas J, Chen YF. ANP signaling inhibits TGF-beta-induced Smad2 and Smad3 nuclear translocation and


APPENDIX

INSTITUTIONAL REVIEW AND ANIMAL CARE AND USE COMMITTEE

APPROVAL FORMS
NOTICE OF APPROVAL

DATE: December 10, 2008

TO: Yiu-Fai Chen, Ph.D.
    ZRB-1008 0007
    FAX: 934-0424

FROM: Judith A. Kapp, Ph.D., Chair
      Institutional Animal Care and Use Committee

SUBJECT: Title: Atrial Natriuretic Peptide and Receptor Genes in Hypoxia
         Sponsor: NIH
         Animal Project Number: 0812076007

On December 10, 2008, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>B</td>
<td>150</td>
</tr>
<tr>
<td>Mice</td>
<td>A</td>
<td>150</td>
</tr>
<tr>
<td>Rats</td>
<td>B</td>
<td>120</td>
</tr>
</tbody>
</table>

Animal use is scheduled for review one year from December 2008. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 0812076007 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7062.
MEMORANDUM

DATE: October 14, 2008

TO: Yiu-Fai Chen, Ph.D.
    ZRB-1008 0007
    FAX: 934-0424

FROM: Judith A. Kapp, Ph.D., Chair
    Institutional Animal Care and Use Committee

SUBJECT: NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.

The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on October 14, 2008.

Title of Application: ANP Modulates Cardiac Remodeling Via Cardiac Fibroblasts
Fund Source: NIH

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW) (Assurance Number A3255-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).