Molecular mechanism of fibrosis and central role of cardiotonic steroids in uremic cardiomyopathy

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FINAL APPROVAL OF DISSERTATION
Doctor of Philosophy in Biomedical Sciences

Molecular Mechanism of Fibrosis and Central Role of Cardiotonic Steroids in Uremic Cardiomyopathy

Submitted by:
Jihad Elkareh

In partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Biomedical Sciences

Examination Committee

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Molecular Mechanism of Fibrosis and Central Role of Cardiotonic Steroids in Uremic Cardiomyopathy

Jihad Victor Elkareh

University of Toledo

2008
DEDICATION

Totus tuus.
ACKNOWLEDGEMENTS

I would like to acknowledge the sacrifice, support, encouragement, guidance, and love of my Mother, Father, and Brother.

I would like to emphasize the commitment, concern, consideration, kindness, cordiality, gentleness, wisdom, support and friendship of Dr. Joseph Shapiro as well as the care, insight, assist, support and friendship of Drs. Sonia Najjar, Zi-Jian Xie, Jiang Liu, and Sandrine Pierre.

I would like to recognize the support of my great friends and co-workers in the laboratory over the years: Amjad Shidyak, David Kennedy, Nasser El-Okdi, Steven Haller, Sleiman Smaili, Adnan Alsaka, Imad Hariri, Vanamala Raju, Liang Wu, Haiping Cai, Sandeep Vetteth, Ajay and Veena Belvadi, Shalini Gupta, Erin Crawford, Yasser Aldhamen, Thomas Blomquist, Carol Woods, Tomasa Guerrero.

I would like to highlight the support, training and companionship of other faculty and mentors from whom I have learned a lot: Drs. Sankaridrug Periyasamy, Larisa Fedorova, James Willey, Bashar Kahaleh, Maurice Manning, Deepak Malhotra, Sadik Khuder, Julie Westerink, Alexei Bagrov, Olga Fedorova, and Sumudra Periyasamy.
TABLE OF CONTENTS

Dedication .......................................................................................................................ii
Acknowledgements .........................................................................................................iii
Table of Contents...........................................................................................................iv
Introduction .....................................................................................................................1
Literature ..........................................................................................................................4
Manuscript 1: Marinobufagenin Stimulates Fibroblast Collagen Production and Causes Fibrosis in Experimental Uremic Cardiomyopathy.................................................20
Manuscript 2: Partial Nephrectomy as a Model for Uremic Cardiomyopathy in the Mouse.................................................................................................................................64
Manuscript 3: Marinobufagenin Induces Increases in Procollagen Expression in a Process Involving Protein Kinase C and Fli-1: Implications for Uremic Cardiomyopathy.........................................................89
Summary ..........................................................................................................................124
Conclusions .....................................................................................................................128
References .......................................................................................................................130
Abstract ..........................................................................................................................146
INTRODUCTION

Cardiac disease is responsible for the high mortality seen in patients suffering from kidney diseases. (1) Impairment of cardiac function is mainly caused by myocardial fibrosis, which is considered the end stage of heart disease. A major biochemical marker of fibrosis would be excessive accumulation of collagen. (3-5)

Transforming growth factor beta (TGF-β) has been established as a key player in the development of fibrosis in many diseases. However, some literature has shown that certain forms of fibrosis, such as atherosclerotic fibrosis, are TGF-β independent. (6) The renin-angiotensin-aldosterone system has been shown to exert profibrotic effects as well. Aldosterone seems to induce cultured fibroblasts to produce more collagen-1 while administration of spironolactone attenuates cardiac fibrosis in vivo.(7) Other studies emphasize the role of the transcription factor Fli-1 in fibrosis. Fli-1 normally competes with Ets-1 in a Sp-1 dependent manner, shifting the balance between stimulating and repressing the Col 1A2 promoter. (2) Jinnin et al showed that the protein kinase PKCδ regulates the expression of collagen-1 through changing the equilibrium between Ets-1 and Fli-1.(8)

Under these conditions, it has been observed that cardiotonic steroids, which bind to the plasmalemmal Na/K-ATPase, accumulate in renal failure. These steroids have been shown to specifically inhibit the sodium pump, as well as circulate at higher levels in patients with kidney disease.
In patients suffering from uremic cardiomyopathy, marinobufagenin (MBG), a cardiotonic steroid, has been established as a natriuretic hormone that increases Na excretion when renal elimination of Na is impaired.

Our laboratory has observed that increases in circulating cardiotonic steroids were both necessary and sufficient to induce systemic oxidant stress, cardiac hypertrophy, and diastolic dysfunction seen with experimental uremia. More importantly, administration of MBG for 4 weeks caused comparable increases in plasma MBG as did the partial nephrectomy at 4 weeks. On the other hand, immunization against MBG attenuated the cardiac hypertrophy, impairment of diastolic function, systemic oxidant stress, and cardiac fibrosis seen with partial nephrectomy.

Therefore, based on data from our laboratory as well as the extensive literature concerning fibrosis, we propose the central hypothesis: Circulating cardiotonic steroids bind to the Na/K-ATPase and produce increased collagen production in a process involving Fli-1, and PKCδ as part of the resultant signaling cascade. This signaling cascade plays a crucial role in the pathogenesis of cardiac fibrosis, a major phenotype of chronic renal failure.

In order to investigate this central hypothesis, we first examined whether cardiotonic steroids, particularly MBG, signal through the Na/K-ATPase, directly stimulating cardiac fibroblast collagen production. Next, the partial nephrectomy model in the rat, which extensively simulates experimental uremia with cardiac abnormalities as seen in renal
failure, was applied to the mouse, which we postulated would yield similar results.

Employing this model, we show the relevance of Fli-1 in the negative regulation of collagen, thus revealing the molecular mechanisms by which cardiotonic steroids contribute to the cardiac fibrosis present in uremic cardiomyopathy.
Epidemiology of Cardiovascular Disease in Chronic Kidney Disease

Chronic kidney disease has been identified as a major risk factor for cardiovascular disease. Because it is estimated that 20 million adults suffer from chronic kidney disease in the United States, Medicare is expected to spend 28 billion dollars by 2010 in an effort to treat this disease.\(^{(26,27)}\) There is a vicious cycle between the kidney and the heart which led many scientists and doctors to term the relationship between these two vital organs as “cardiorenal syndrome”.\(^{(26, 28, 29)}\) Other studies showed that the mortality rate is still high in patients with end stage renal disease (ESRD) including patients on Hemodialysis (HD). In fact, 25-50\% of patients under dialysis develop cardiac failure, and more than 50\% of the mortalities result from cardiac related causes.\(^{(30, 31, 114)}\) These findings suggest that HD can not replace normal renal function and this tightly correlates renal disorders with cardiac malfunctions. Dialysis is capable of inducing myocardial ischemia thus leading to heart failure.\(^{(32, 33)}\) It is believed to be pro-arrhythmogenic since it induces arrhythmias and causes increase in QT interval and QT dispersion.\(^{(34)}\) In addition to the arrhythmia, diastolic dysfunction and left ventricular hypertrophy are very common in patients with ESRD, despite treatment with HD.\(^{(35-39)}\) In 1998, the National Kidney Foundation issued a report emphasizing the high risk of cardiovascular disease in chronic kidney disease patients.\(^{(30)}\) Clinically, this is called uremic cardiomyopathy and is characterized by diastolic dysfunction and left ventricular
hypertrophy. At present, the molecular basis of uremic cardiomyopathy is poorly understood. It is known to be characterized by systemic oxidant stress, diastolic dysfunction, and left ventricular hypertrophy, and cardiac fibrosis. Anemia, hypertension, parathyroid hormone, the renin-angiotensin-aldosterone system (RAAS), and lipid abnormalities have been implicated as potential pathogenetic factors in uremic cardiomyopathy. Treatment with recombinant erythropoietin or a parathyroidectomy has shown regression in left ventricular hypertrophy one of the major symptoms of uremic cardiomyopathy.

Components of Fibrosis

Impairment of cardiac function is mainly caused by myocardial fibrosis, and is considered the end stage of heart disease with symptoms including cardiac dilatation, reduction in contractility and relaxation, with progressive structural and functional alterations. Fibrosis is the result of an imbalance between synthesis and degradation of the extracellular matrix (ECM). This disparity leads to abnormal remodeling of ECM. In fact, extensive research has shown a deposition of collagen, a key marker of fibrosis, in the left ventricular causing hypertrophy of the heart and in some cases an increase in blood pressure. The ECM or connective tissue, is a structural entity that supports the cell, which is composed of three major classes of biomolecules: structural proteins (collagen and elastin), specialized proteins (fibrillin, fibronectin and laminin), and proteoglycans.
Matrix metalloproteases (MMPs) also called matrixins, which are responsible for degradation and remodeling of the ECM. They have been classified in six subgroups: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other MMPs.(44)

Collagen, the major component of ECM, has a balanced turnover (synthesis and degradation) that is estimated to be 80-120 days in cardiac fibroblasts. The turnover of collagen is regulated by MMPs and their inhibitors TIMPs (tissue inhibitors of metalloproteases). On a regular basis, fibroblasts control collagen turnover, nevertheless during pathologic circumstances, fibroblasts are transformed into myofibroblasts. These myofibroblasts can derive from two other sources: the bone marrow of stem cells, where they are referred as fibrocytes and the epithelial cells in a process know as EMT (epithelial- mesenchymal transition). (45-47) The transition from fibroblasts to myofibroblasts is mainly regulated by TGF-β and the renin-angiotensin-aldosterone system (RAAS)(45). When myofibroblasts are activated via its respective stimulants, MMPs are produced as a result (46). According to Berk et al, an imbalance in the ratio of MMPs to TIMPs might explain the left ventricular dilatation and the reduced ejection fraction in congestive heart failure. Therefore, left ventricular hypertrophy can be seen where there is ECM accumulation in the heart, whereas a transition to systolic failure can be observed in hearts that have an increase in ECM degradation.(45)

Once an injury occurs, the regenerative/wound healing phase takes place, and injured cells are replaced by cells from the same type in a progression which leaves no evidence of scarring. On the other hand, the repair process might occur in a different way such as
the replacement of normal parenchymal tissue by connective tissues would potentially leave a permanent scar from the substantial remodeling of the ECM. “Out of control wound healing” is referred at this point to as fibrosis or fibroplasia, which can be preceded by inflammation, although it is not driven by it at all times. (46) This in part explains why anti-inflammatory drugs alone can not treat fibrosis efficiently. Before the scarring stage, when fibrosis is reversible, the molecules of choice that have been used so far were MMP inhibitors. Unfortunately, these molecules showed many side effects such as tendonitis which limited the pharmacological development of MMP inhibitors. (45, 46)

Pathogenetic Mechanism in Fibrosis

TGF-β/BMP/Smad System:

Increases in collagen formation in the tissue could be explained as a combination of transforming growth factor (TGF-β- dependant and TGF-β− independent) mechanisms. Orlandi and colleagues showed that ischemia associated fibrosis appears to be TGF-β dependent, while aging related fibrosis and atherosclerosis appears to be TGF-β independent. (6) TGF-β stimulation of collagen-1 production involves a very well characterized signal transduction cascade, which involves the essential participation of Smad proteins. (48-58) Bone morphogenic proteins (BMP) which also involves Smad proteins can stimulate fibrosis. The TGF-β/BMP/Smad system has been well studied over
the years and is believed to be involved in collagen regulation. TGF-β binding to TGF-β receptors leads to full assembly of the receptors which can activate Smad2, and Smad3. Inhibitory Smads, which are stimulated by several cytokines, antagonize the assembly of the TGF-β receptor complex. TGF-β signals through Smad2 and 3 whereas BMPs signal through Smad1, 5, and 8. Assembly of Smad4 (co-Smad) with active or phospho-Smad2 or 3 enters the nucleus and acts as a transcription factor. (51) Although multiple aspects of collagen synthesis are affected by the TGF-β/BMP/Smad system, transcriptional regulation of collagen synthesis constitutes a major component of this important pathway. (59)

**Renin-Angiotensin-Aldosterone System (RAAS):**

Every component of RAAS exerts profibrotic effects on the cells. For instance, renin, independently of its enzymatic action to enhance angiotensin II synthesis, increases the synthesis of collagen I and fibronectin. (60) Angiotensin II enhances fibrosis directly through the angiotensin II type 1 receptors, and indirectly through the induction of TGF-β, which is considered the major RAAS hormone responsible for cardiac fibrosis. Likewise, angiotensin II induces the differentiation of fibroblasts to myofibroblasts in a pathway involving ROS and the activation of p38 MAPK, and in an additional pathway involving tyrosine kinase mediated by c-Src. Finally, the mineralocorticoid steroid hormone, aldosterone, has been a focus of recent investigation, especially in light of
spironolactone and eplerenone, two of its inhibitors. Susic et al showed that long-term therapy with the mineralocorticoid receptor antagonist eplerenone reduced myocardial fibrosis, and improved left ventricular function and coronary hemodynamics in spontaneous hypertensive rats. In other literature, supraphysiological levels of aldosterone seem to induce cultured fibroblast to produce more collagen-1 while administration of spironolactone attenuate cardiac fibrosis in vivo.(7) Consequently, therapies that target the TGF-β/BMP/Smad system or RAAS might be effective in the treatment of fibrosis in congestive heart failure and other heart diseases.

Fli-1/Ets-1 System:

Fli-1 (Friend leukemia virus integration 1) is member of the Ets (E26 Transformation Specific) transcription factor family of proteins and belongs to the ERG subfamily.(61) All Ets transcription factors are defined by a winged helix-turn-helix DNA binding domain (ets domain) that is specific to DNA element containing a purine-rich sequence GGAA/T.(62) In the Fli-1 sequence, the 3’ ets domain (amino acids 277-360) is responsible for sequence specific DNA-binding activity, whereas the 5’ ets domain (amino acids 121-196) and the FLS (Fli-1 specific region) (amino acids 205-292) contain the sequence responsible for transcriptional activation.(61) An analysis of the secondary structure of Fli-1 showed the presence of helix1-loop-helix2 (H-L-H) structures in the 5’ and 3’ ets domains and revealed the presence of turn-loop-turn (T-L-T) structures in the FLS domain as well as the CTA (carboxy-terminus) (amino acids 402-452)(61) Fli-1 was
commonly found as a site for retroviral integration in Friend-virus induced erythroleukemias. (61, 63) Fli-1 was also found highly expressed in vascular endothelial cells and hematopoietic cells. (64)

After the cloning of mouse Fli-1, the human Fli-1 gene was isolated by sequence homology. The sequence analysis showed a high level of conservation between the rodent and human Fli-1. (61, 65, 66) While Fli-1 is on chromosome 9 in the mouse and on chromosome 11q23 in the human, it is located 240kb from the Ets-1 locus on both chromosomes. Fli-1 encodes for a 452aa protein: p51 and a 419aa protein: p48. (61, 63, 66) According to Sarrazin et al, the synthesis of the 51 and 48 isoforms were initiated from codons AUG +1 and AUG +100 of Fli-1 respectively. (67)

It is well known that in the human body there is always equilibrium between stimulating and repressing factors. In particular, in organ fibrosis there is a competition between pro-fibrotic and anti-fibrotic signaling pathways. Czuwara-Ladykowska and colleagues emphasize the role of the Fli-1 in fibrosis: Fli-1 normally competes with Ets-1 in a Sp-1 dependent balance between stimulating and repressing the Col 1A2 promoter. (2) Most of the work has been done in dermal fibroblasts showing that Ets-1 and Fli-1 have antagonistic effects on the collagen gene promoter and they compete with each other in the regulation of this promoter. (figure 1) (2)

In recent years, a lot of research has been conducted by Kahaleh and colleagues on the role of Fli-1 as a suppressor of collagen transcription in scleroderma fibroblasts. In fact in systemic sclerosis or scleroderma, which is an autoimmune disease, there is an abnormal
deposition of collagen on the skin. (68, 69) Studies have shown that in scleroderma patients, Fli-1 gene is repressed due to epigenetic mechanisms notably a DNA methylation and a histone deacetylation at the Fli-1 gene. (69). On the other hand, Trojanowska et al reported a reversible acetylation of Fli-1 on the lysine residue due to TGF-β stimulation in human dermal fibroblasts. PCAF (p300/CBP-associated factor), which is a transcription factor that has histone acetyltransferase activity, interacts with Fli-1 on lysine 380 leading to Fli-1 acetylation. (62) Like most of the acetylation that occurs on the lysine residue, an impairment of Fli-1 DNA-binding to the Col 1A2 promoter resulted in a decrease in the inhibitory effect Fli-1 has on Collagen. (62, 70) According to the authors, the TGF-β/ Smad pathway seems to be responsible for the dissociation of Fli-1 from the collagen promoter, which explains the fibrosis seen in systemic sclerosis. (62)

![Figure 1: Schematic suggesting competition between Fli-1 and Ets-1 at the level of the Col1a2 promoter to determine whether transcription is repressed or stimulated. HDAC1= histone deacetylase 1. Adapted from reference. (2)](image-url)
PKC isoforms and Role in Fibrosis:

In the late seventies, the protein kinase C (PKC) has been identified and since then extensive work has been done on this family of serine/threonine kinase especially its role in exocytosis, growth, differentiation, and modulation of ion channels and receptors.(71) The PKC family can be divided into four groups: conventional/classical PKCs (PKCα, PKCβ1, PKCβ2, and PKCγ), novel PKCs (PKCδ, PKCε, PKCη, and PKCθ), atypical PKCs (PKCζ and PKCλ/τ), and the recently discovered PKCs (PKCμ and PKCν).(72, 73) These twelve isozymes are implicated in many cellular responses in normal lung function such as: permeability (PKCα), contraction (PKCβ2), migration (PKCδ), hypertrophy (PKCη), proliferation (PKCε) apoptosis (PKCζ), and secretion (PKCν). (73)

Recent literature suggests a mechanism of action that involves phospholipase C, which performs a catalytic mechanism and generates inositol triphosphate (IP₃) and diacylglycerol (DAG), which then cause PKC enzymes (key targets of DAG) to recognize and phosphorylate human EGFR at the threonine-654, (74) inducing the internalization of EGFR to be recycled to the plasma membrane.(75)

On the other hand, six isozymes have been found to have different roles in the heart. PKCε and PKCδ are both activated by the same receptor Gaq, however PKCδ preferentially activates p38, MAPK, and JNK while PKCε activates p42/44 MAPK. In
fact, PKCε is considered being the PKC that protects the heart from ischemic damage. Many studies showed evidence that PKCε is required to produce protection from ischemic damage in animal models like mice and in isolated myocytes. Also, ψεRACK, a selective agonist for PKCε, could be used in therapeutics in treating ischemic heart disease in humans.(73) In contrast, PKCδ is associated with increased collagen deposition and impairment of diastolic function. There is a possible correlation between PKCε and PKCδ expression levels. When PKCε expression levels are low such as in a knock out animal model, PKCδ expression levels appear to compensate this deficiency leading to upregulation of PKCδ which is closely associated with heart fibrosis.(72)
The Protein Kinase C family of proteins mediates the specific activation of a variety of transcription factors like NFkB and Ap1, but little is known whether it can be involved in the activation of Ets Family, and specifically Fli-1. Nevertheless, Jinnin et al showed that PKCδ regulates the expression of collagen-1 through shifting the balance of Ets-1 and Fli-1.(8) Moreover, Jimenez et al. proposes that PKCδ inhibitors could suppress fibrosis, indicating that PKCδ participates in the upregulation of collagen gene transcription. (25) In addition, they suggest that the activity of several Na/K-ATPase inhibitors such as Noradrenaline and phorbol 12-myristate 13-acetate (PMA) is stimulated by PKC activation. (76) We noticed that the Fli-1 relationship to collagen metabolism and fibrosis in general has been carefully examined in dermal fibroblasts, and very recently in lung fibroblasts (42), but little is known about its role in creating fibrosis in other organs like the heart and the kidney.
**Na/K-ATPase Function**

First discovered in 1957, (77) the Na/K-ATPase has been identified as an enzyme present in most cells. (78) From the outer medulla of pig kidneys, Hebert et al. showed the the Na/K-ATPase structure was elucidated via electron crystallography at 9.5 A. (79) It belongs to the P-Type ATPases which contain Na/K-ATPase, H/K-ATPase, SERCA (sarcoplasmic endoplasmic reticulum calcium ATPase) and PMCA (plasma membrane calcium ATPase). Among these ion pumps, Na/K-ATPase is the only one that has the ability to bind cardiotonic steroids such as ouabain and marinobufagenin. (80) The P-Type ATPases consists of two monoconvalently linked $\alpha$ and $\beta$ subunits, which are essential for the assembly of the functional enzyme. As for the $\alpha$ subunit (112 KDa) it is considered the catalytic subunit because it contains the ATP and binding sites for digitalis and other ligands. (81-83) There is 78% similarity among all $\alpha$ isoforms ($\alpha_1$, $\alpha_2$, $\alpha_3$, $\alpha_4$), and the $\alpha_2$ and $\alpha_3$ isoforms are expressed in neuronal tissue, skeletal muscle, and cardiac myocytes. The $\alpha_1$ isoform is found in all cells which makes it difficult to study the other isoforms in a specific organ. (84, 85) The main function of the Na/K-ATPase is to catalyse the active exchange of three cytoplasmic Na$^+$ for two extracellular K$^+$ across the plasma membrane via hydrolysis of ATP. (78, 86) In our laboratory we found a rise in cardiac tissue calcium concentrations, thus causing increases in myocardial contractility when the Na/K-ATPase is inhibited with digitalis(22). Additional studies showed that
the enzyme is also a signal transducer involved in regulation of multiple secondary messengers, gene-regulatory pathways, and pathways including activation of Src, EGFR, Ras, PKC, MAPKs, and intracellular ROS production. (10-19, 83, 87, 114) Since it communicates with the nucleus, the pump plays a role in the regulation of gene expression and cell proliferation. When ouabain binds to the pump, the Na/K-ATPase interacts with neighboring membrane proteins and organizes a cytosolic cascade of signaling complexes to send messages to other intracellular organelles. This includes activation of Src, Ras, and MAPKs, transactivation of EGFR, increased production of ROS, intracellular $[Ca^{2+}]$ and myocardial contractility. (10, 12, 14, 15, 20, 114) This model has been shown in different cell lines such as neonatal rat cardiac myocytes, smooth muscle cells, and kidney tubular cells. (11, 13, 17, 24, 92, 93, 96)

There has been recently an interest towards endocytosis of the Na/K-ATPase and the role it could play in signal transduction through the pump. In fact, dopamine and parathyroid hormone stimulation induces endocytosis of the plasmalemmal Na/K-ATPase. (99, 100) Depending on the cell type, this endocytosis is related to either PKC or PKA activation and requires phosphorylation of specific amino acids in the $\alpha$-chain of the Na/K-ATPase. (101, 102). Liu et al have demonstrated that cardiotonic steroids could stimulate internalization of the Na/K-ATPase in proximal tubular cells in vivo and in porcine proximal tubular (LLC-PK1) cells in vitro (13, 21, 22, 24, 96, 114). Moreover, internalization of the Na/K-ATPase was associated with accumulation of both the cardiotonic steroids, and the Na/K-ATPase itself (13). This process was found to require intact caveolar architecture, via caveolin1, a scaffold protein that belongs to this caveolar...
compartment. Cardiotonic steroids stimulate a clathrin-dependent endocytosis pathway which translocates the Na/K-ATPase to intracellular compartments under the activation of Src. (24).

Circulating cardiotonic steroids and mammalian physiology:

For more than two centuries a cardiotonic steroids, have been used to treat congestive heart failure. In 1953, Szent-Györgyi and Schatzmann revealed that cardiotonic steroid are specific inhibitors of the sodium pump and that the digitalis receptor is the Na/K-ATPase. (103) Before ouabain, marinobufagenin or digoxin were shown to be involved in the sodium pump, researchers referred constantly to ‘‘3rd factor’’ a substance that inhibits the Na/K-ATPase. (104, 105, 114) In the early 80s doctors found increased levels of digoxin and some antibodies in chronic renal failure patients not taking the digoxin medication, which they later termed ‘‘false positive’’ digoxin. (106, 107, 114) Since then extensive work has been done to find the involvement of cardiotonic steroids in uremic cardiomyopathy and its symptoms like hypertension and hypertrophy of the heart. Extensive researches have been shaped through ouabain which is derived from plant tissue and is considered the cardiotonic steroids of choice to study in most laboratories. A compound that is immunologically quite similar to plant derived ouabain (ouabain like compound or OLC) can be detected in a number of mammalian tissues, like in the hypothalamus of cattle. OLC is an optical isomer of ouabain derived from plants. (108,
114) Ouabain (figure 2) exhibits high affinity for the $\alpha_2/\alpha_3$ Na/K-ATPase isoforms. Since the tubular cells of the kidney in mammals express mainly the $\alpha_1$ isoform, ouabain turn out to be insensitive in this case. Furthermore, endogenous ouabain levels were not increased by chronic administration of high salt diet or by acute saline plasma volume expansion.(109)

Not all the hypertension effects could be attributed to OLC; therefore researchers started thinking of another sodium pump inhibitor family. Marinobufagenin (3, 5-dihydroxy-14, 15-epoxy bufodienolide) (figure 3) which is a member of the bufodienolides family of the Na/K-ATPase inhibitors has been later identified. Bufodienolides differ from cardenolides in having a doubly unsaturated six-membered lactone ring. Marinobufagenin in its endogenous form is a potent vasoconstrictor and has been extracted from some amphibians (certain species of toads). (110, 111) Marinobufagenin-like compounds were later found in plasma and urine of humans, dogs, mice, and rats. MBG is considered a natriuretic hormone that increases Na excretion when renal elimination of Na is impaired by loss of renal function and this is seen in patients suffering from uremic cardiomyopathy. In addition, patients under a high salt diet were observed to have increased MBG in the system to eliminate the excess of sodium. Although these cardiotonic steroids circulate with concentrations in the high pM to low nM range, they are able to inhibit the sodium pump and have very significant physiological effects. These hormones showed increases in the growth of rat cardiac myocytes grown in culture. (112, 114) We have shown substantial increases in blood pressure by only 4 weeks with MBG infusion. (9) Although ouabain and MBG have very
similar inhibitory effects on the pump, it is believed that their impact on fibrosis in the heart and other tissues may vary depending on the amount of administration of the cardiotonic steroid and its duration.

It has been established that in an acute salt loading, endogenous ouabain acts essentially as a neurohormone in the hypothalamus to activate central and adrenal angiotensin II which in turn stimulate the sympathetic nervous system through norepinephrine that triggers increase plasma levels of MBG. Hence, the end result involving ouabain and MBG will produce a 35 mmHg rise in the arterial blood pressure and a 45% inhibition of the Na/K-ATPase. (41, 109)

Many speculated that age associated increases in systolic blood pressure in older humans is correlated to circulating cardiotonic steroids. Recently, it has been shown that MBG excretion is associated to increases in sodium excretion and is inversely related to age or age-dependent increases in salt sensitivity. On the other hand, endogenous ouabain excretion did not correlate with age, sodium excretion or salt sensitivity.(113) We can conclude that both cardenolides and bufadienolides are involved in the regulation of hypertension.
Figure 2: Chemical structure of Ouabain

Figure 3: Chemical structure of Marinobufagenin
Marinobufagenin Stimulates Fibroblast Collagen Production and Causes Fibrosis in Experimental Uremic Cardiomyopathy

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\textit{Hypertension. 2007;49:215-224.}
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Short Title: Cardiotonic steroids stimulate collagen production

Key Words: Cardiomyopathy; Renal Failure, TGF-β, Cardiotonic Steroids; Reactive Oxygen Species; Fibrosis
Abstract

Introduction: We have observed recently that experimental renal failure in the rat is accompanied by increases in circulating concentrations of the cardiotonic steroid, marinobufagenin (MBG), and substantial cardiac fibrosis. We performed the following studies to examine whether MBG might directly stimulate cardiac fibroblast collagen production.

Methods: In vivo studies were performed using the 5/6th nephrectomy model of experimental renal failure (PNx), MBG infusion (MBG), PNx after immunization against MBG, and concomitant PNx and adrenalectomy. Physiological measurements with a Millar catheter and immunohistochemistry were performed. In vitro studies were then pursued with cultured isolated cardiac fibroblasts.

Results: We observed that PNx and MBG increased MBG levels, blood pressure, heart size, impaired diastolic function, and caused cardiac fibrosis. PNx after immunization against MBG and concomitant PNx and adrenalectomy had similar blood pressure as PNx but less cardiac hypertrophy, diastolic dysfunction, and cardiac fibrosis. MBG induced increases in procollagen-1 expression by cultured cardiac fibroblasts at 1 nM concentration. These increases in procollagen expression were accompanied by increases in collagen translation and increases in procollagen-1 mRNA without any demonstrable increase in procollagen-1 protein stability. The stimulation of fibroblasts with MBG could be prevented by administration of inhibitors of tyrosine phosphorylation, Src activation, epidermal growth factor receptor transactivation, and N-acetyl cysteine.
**Conclusion**: Based on these findings, we propose that MBG directly induces increases in collagen expression by fibroblasts, and we suggest that this may be important in the cardiac fibrosis seen with experimental renal failure.
Introduction

Cardiac disease is directly responsible for the extremely high morbidity and mortality seen in patients with end-stage renal disease.\(^1\) Clinically this cardiac disease of renal failure, also called uremic cardiomyopathy, is characterized by diastolic dysfunction and left ventricular hypertrophy in the setting of systemic oxidant stress.\(^2\) Although a number of potential mechanisms, such as elevations in parathyroid hormone, hypertension, and anemia, have been implicated as contributors to the cardiac disease seen in this setting, its pathogenesis is still a bit unclear.

On this background, we have demonstrated previously that the cardiotonic steroid marinobufagenin (MBG), signaling through the Na/K-ATPase, is directly responsible for many features of experimental uremic cardiomyopathy induced by partial nephrectomy (PNx) in the rat.\(^3\) Specifically, we noted that both rats subjected to PNx, as well as rats given MBG supplementation by minipump, developed considerable cardiac hypertrophy and fibrosis by four weeks, whereas rats immunized against MBG and subsequently subjected to PNx had attenuation of these changes. From these data, we formulated the hypothesis that MBG might directly induce cardiac fibroblasts to produce collagen, thus producing much of the cardiac fibrosis seen with experimental renal failure. To test this hypothesis and to determine the molecular basis by which this occurred, the following studies were performed.
Methods

Animals
Male, Sprague–Dawley rats were used for all of the studies. All of the animal experimentation described in the article was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals using protocols approved by the Medical University of Ohio Institutional Animal Use and Care Committee.

Experimental Groups
Briefly, Sprague–Dawley rats weighing ≈ 250 g at the time of surgery were subjected to either sham surgery with no MBG infusion (Sham), sham surgery with placement of a minipump infusing MBG at 10 μg/kg per day (MBG), PNx, and PNx after immunization against MBG (PNx-IM). MBG of extremely high purity (>99%) was isolated from the venom of *Bufa marinus* by Kennedy et al.³ In addition to these maneuvers, a group of PNx animals was subjected to adrenalectomy as well (PNx-ADx).

The heart weight normalized to body weight, left ventricular hemodynamics (eg, τ value, slope of regression line fit to end diastolic pressure versus end diastolic volume generated by inferior vena cava occlusions, all determined with a Millar catheter), plasma [MBG] (determined after extraction on a C-18 column using DELPHIA as described previously³), aldosterone (determined with ELISA kit 10004377, Cayman Chemical) and cardiac immunohistochemistry (vida infra) were assessed 4 weeks after surgery.
Isolated Cardiac Fibroblasts

Preparation of adult rat cardiac fibroblasts was performed as described previously by Brilla et al.\textsuperscript{4} with modifications.

Western Blot Analysis

Western blot analysis was performed on protein isolated from tissue homogenates, cell culture whole cell lysates, or nuclear extracts as described previously.\textsuperscript{2}

Collagen Synthesis

Collagen synthesis rates were determined by the method of Nishida et al.\textsuperscript{5} with modifications.

Quantitative Measurement of Collagen-1 mRNA

Standardized RT-PCR was used to measure gene expression, with GAPDH transcript used as the housekeeping gene, as reported previously.\textsuperscript{6}

Statistical Analysis and Expanded Methods

Statistical analysis and details regarding experimental groups and specific measurements are provided in an online supplement (http://hyper.ahajournals.org).
Results

Effect of Experimental Renal Failure and MBG on Blood Pressure, Cardiac Hemodynamics, and Fibrosis

In the current in vivo studies (data summarized in the Table), we observed that MBG levels were increased in PNx- and MBG-treated rats compared with sham-operated controls. We also saw that both PNx and MBG rats had higher systolic blood pressure than controls and that PNx-IM rats had statistically similar systolic blood pressure values as seen with PNx. Using the Millar pressure/volume sensor catheter rather than echocardiography in our previous report, we observed that PNx induced decreases in end systolic volume and end diastolic volume, as well as increased ejection fraction compared with sham-operated controls. The end systolic volume and end diastolic volume were greater, and the ejection factor values were lower in PNx-IM as compared with PNx. Active relaxation assessed by $\tau$ was found to be impaired by both PNx and MBG compared with sham-operated controls, with PNx-IM showing lower values than PNx. Using pressure volume loops generated during vena cava occlusions (representative tracings shown in Figure 1a), we noted that the end diastolic pressure volume relationship (an inverse measurement of passive compliance) was increased in PNx- and MBG-treated animals compared with controls, whereas PNx rats had a lower end diastolic pressure volume relationship than PNx. Both PNx and MBG treatment increased the heart weight/body weight ratio compared with sham-operated controls, whereas PNx-IM animals had lower values than PNx (Table). Examining the ventricular myocyte cross-
sectional area determined on trichrome images, we noted that PNx and MBG infusion both induced marked increases, whereas the myocyte cross-sectional area in PNx-IM was considerably smaller than that seen with PNx alone (Figure 1b).

Analyzing the immunohistochemistry results, heart tissues from rats subjected to MBG and PNx showed marked increases in collagen-1 and α smooth muscle actin staining. Immunization against MBG attenuated these increases (Figure 1c and 1d). Western blot analysis confirmed that PNx and MBG had 2 to 2.5 times the expression of procollagen-1 and α smooth muscle actin seen with sham-operated controls, whereas PNx-IM expression of both procollagen-1 and α smooth muscle actin was substantially less than that seen with PNx (Figure 1e and 1f).

To determine the molecular mechanism underlying this fibrosis, we examined the expression of several proteins important in fibroblast activation. Specifically, we examined tissue levels of transforming growth factor (TGF)-β, Smad 2/3, and Smad 4, as well as pSmad 2/3. We did not detect significant differences among the experimental groups in the cardiac expression of these proteins (Figure 2a–2d).

A separate group of animals (N=11) was also subjected to PNx-ADx with physiological replacement of glucocorticoids and aldosterone.7 These animals developed a similar degree of hypertension compared with PNx but were noted to have much lower plasma MBG and aldosterone levels, as well as substantially lower heart/weight body weight ratio compared with PNx alone (Table). Moreover, these animals subjected to PNx-ADx had almost no evidence for cardiac fibrosis based on trichrome staining or
immunohistochemistry staining for collagen-1 or α smooth muscle actin (see the online data supplement).

**Effect of Cardiotonic Steroids on Fibroblast Collagen Expression**

To further examine the molecular basis of this cardiac fibrosis, isolated cardiac fibroblasts were subjected to increasing doses of MBG ($10^{-10}$, $10^{-9}$, and $10^{-8}$ M). After 24 hours of exposure to $10^{-9}$ and $10^{-8}$ M MBG, procollagen content determined by Western blot was increased ≈2 fold (both $P<0.01$; Figure 3a). This phenomenon was not specific for MBG; other cardiotonic steroids also induced similar increases in procollagen content (Figure 3b). Of interest, the threshold for effect for MBG seemed to be between $10^{-10}$ and $10^{-9}$ M, whereas for ouabain, which circulates at similar concentrations in uremic rats, the threshold was ≈10 times higher (ie, between $10^{-9}$ and $10^{-8}$ M). For both MBG and ouabain, the threshold for inducing collagen expression was log units below the doses necessary for detectable effects on 86Rb uptake in these cells (Figure 3c). In parallel studies examining radiolabeled proline incorporation into collagen, we observed that $10^{-9}$ and $10^{-8}$ M MBG induced significant increases in both proline incorporation into total protein, both matrix and supernatant. Using collagenase digestion, we observed that the vast majority of the proline incorporation was into collagen (Figure 3d). Using standardized RT-PCR, we observed a doubling of mRNA for collagen-1 at 24 hours in response to 10 nM of MBG (Figure 3e). However, we did not detect any increases in
procollagen stability (determined by examining procollagen-1 expression after exposure to cycloheximide) in response to this concentration of MBG (Figure 3f).

Effect of Inhibition of Na/K-ATPase Signaling on MBG-Stimulated Collagen Expression

To examine whether cardiotonic steroids induced collagen synthesis by signaling through the Na/K-ATPase, we performed the following studies. First, we used pharmacological antagonism at several steps in the Na/K-ATPase cascade. Specifically, we used pharmacological antagonism of Src activation with PP2, nonspecific tyrosine kinase inhibition with herbimycin, inhibition of EGFR transactivation with AG1478, and nonspecific antioxidant administration with N-acetyl cysteine. Each of these maneuvers prevented MBG stimulation of collagen synthesis (Figure 4a). To confirm these data, we also examined radiolabeled proline incorporation in response to MBG in the presence and absence of either PP2 or N-acetyl cysteine. As was the case for procollagen expression, both PP2 and N-acetyl cysteine prevented increases in proline incorporation into collagen in the primary fibroblast cultures (Figure 4b). Next we performed studies in the SYF and SYF+ cells (details available in the online supplement). SYF+ cells responded to MBG and ouabain in a very similar way as the primary cardiac fibroblast cultures with respect to upregulation of procollagen expression, whereas the SYF cells had essentially no response to either MBG or ouabain (Figure 4c).
Relationship between TGF-β and MBG-Stimulated Collagen Production

To further examine the molecular mechanisms by which cardiotonic steroids induce collagen production in fibroblasts, we examined the effects of MBG on TGF-β expression, as well as the expression of Smad 2/3, Smad 4, and pSmad 2/3. As was the case for the in vivo experiments described earlier, we did not observe significant changes in TGF-β, Smad 2/3, Smad 4, or pSmad 2/3 expression in vitro (Figure 5a). Next, we examined whether TGF-β induced collagen production and whether there was synergism between TGF-β and MBG. In the primary cultured cells, we saw similar effects of TGF-β (5 ng/mL) on procollagen expression as observed with cardiotonic steroids; however, we did not note any synergism between TGF-β (5 ng/mL) and MBG (10 nM). However, it is important to point out that we never completely serum starve the primary cultures, and because serum is always present, some TGF-β is always present.

To address this further, we also examined the effect of the TGF-β receptor antagonist, SB431542, on MBG stimulated collagen production. Interestingly, SB431542 at 100-μmol/L concentration did not reduce procollagen expression below baseline on our Western blots (Figure 5b) but did decrease radiolabeled proline incorporation below that seen with control cells (Figure 5c). The SB431542 completely blocked both TGF-β and MBG (10 nM) stimulation of collagen expression and radiolabeled proline incorporation (Figure 5b and 5c).
Discussion

Cardiac fibrosis is an important component of many cardiomyopathies, and it is a very characteristic component of uremic cardiomyopathy. Our group and others have observed that MBG and other cardiotonic steroids induce a signal transduction cascade through the plasmalemmal Na/KATPase residing in caveolae, which results in activation of Src, transactivation of the EGFR, generation of reactive oxygen species, and, ultimately, activation of p42/44 mitogen-activated protein kinase. Interestingly, a number of clinical situations associated with cardiac fibrosis other than renal failure are associated with increased circulating concentrations of cardiotonic steroids (eg, hypertension, primary hyperaldosteronism, and congestive heart failure). Although it is preliminary to discuss the possible relevance of our findings to cardiomyopathies other than renal failure, we should point out that Ferrandi et al have observed that antagonism of endogenous cardiotonic steroids with PST 2238 ameliorates hypertension, as well as cardiac hypertrophy in Milan hypertensive rats.

In the current study, we confirmed that PNx and MBG treatment induce similar but not identical phenotypic changes in hemodynamics and cardiac morphology. It is quite likely that some factors other than MBG contribute to the phenotypic changes seen in PNx. That said, both PNx-IM and PNx-ADx, which reduce circulating MBG, substantially attenuate the cardiac functional and morphological changes without significantly affecting blood pressure. We should point out that experiments in the PNx-ADx model were performed because we reasoned that as adrenal cells grown in culture
seem to make MBG,\textsuperscript{20,21} it was likely that this procedure would lower the circulating levels of this hormone. However, whereas our data in the PNx-ADx animals support the concept that the adrenal gland is the major (but not the only) site of MBG production in vivo, it is also possible that other hormones made in the adrenal gland modulate MBG production elsewhere. Further work will be necessary to clarify exactly where MBG is produced under normal and pathological conditions.

With these findings implicating MBG in the pathogenesis of cardiac fibrosis, we were particularly interested in the molecular mechanisms underlying the fibrosis. Interestingly, evidence for increases in TGF-\(\beta\) or signaling through the Smad proteins was not evident. We stress that these data do not exclude a role for TGF-\(\beta\) in this process, because earlier increases in these proteins, translocation of the Smads, and/or a permissive role for signaling through this pathway (vida infra) could certainly be present.

Based on these in vivo data, we pursued studies in isolated cardiac fibroblasts. We observed that MBG in physiological concentrations directly stimulated the fibroblasts to produce more collagen. This increase in collagen production was also observed with other cardiotonic steroids, although the threshold concentration seemed to be \(\approx 1\) log unit lower for MBG than for ouabain. We emphasize that the concentration of both MBG and ouabain necessary to stimulate collagen expression was lower for both substances than that needed to appreciably inhibit \({}^{86}\text{Rb}\) uptake. Further evidence for this phenomenon being dependent on signaling through the Na/ K-ATPase was that this increase was prevented by reactive oxygen species scavenging, antagonism, or knockout.
of Src, as well as prevention of EGFR transactivation, maneuvers that we have demonstrated previously to block signal transduction through the Na/K-ATPase signalsome.\textsuperscript{13,14,22–24} We also observed that the increases in collagen production were associated with increases in proline incorporation, as well as increases in mRNA for collagen-1. No increase in procollagen-1 stability could be demonstrated in response to MBG.

Although increases in TGF-\(\beta\) or the Smad proteins were also absent in the fibroblasts treated with MBG, it is important to note that the fibroblasts that we studied were never truly serum starved. In fact, based on a Hyclone web page (http://www.hyclone.com/pdf/atsv19n3.pdf), we would estimate that the fibroblasts were exposed to \(\geq 0.12\) ng/mL of TGF-\(\beta\) even when cultured in the serum-depleted (1\% FBS) medium. This may, in part, explain why SB431542 was so effective in preventing MBG-stimulated collagen production. Working with a similar preparation, Lijnen and Petrov\textsuperscript{25} noted that long incubations (48 hours) and high concentrations of TGF-\(\beta\) (15 ng/mL) were necessary to induce maximal (2 times) increases in collagen production. We should also note that TGF-\(\beta\) blockade with SB431542 actually decreased proline incorporation below baseline, even in the setting of MBG synthesis, although this same pharmacological maneuver only reduced procollagen expression to baseline when measured with Western blot. We suspect that other mechanisms of regulation of collagen synthesis (eg, procollagen stability) might come into play when the TGF-\(\beta\) pathway is interrupted, although we did not explore this point further in the current studies. On
balance, our data argue, albeit preliminarily, against a major role for TGF-β or upregulation of Smad proteins in cardiotonic steroid–induced increases in fibroblast collagen production.

Our data suggest that, in our experimental rodent model, MBG is implicated in the pathogenesis of the cardiac fibrosis, and the concentrations of MBG that develop in this setting, as well as other cardiotonic steroids, have in vitro effects that are consistent with this observation. One issue that immediately comes to mind is whether the clinical use of digitalis might have similar effects. To this question, we would suggest the following possibilities. First, it may be that the free concentrations of digoxin that occur in vivo are not sufficient to induce substantial cardiac fibrosis. Total digoxin levels are typically maintained <2 ng/mL in patients treated with digoxin, a concentration that corresponds with ≈ 2.5 nM concentration. However, only 70% to 80% of the plasma digoxin is free, and the free concentration might fall below the threshold level of digoxin necessary to stimulate human cardiac (or other tissue) fibroblasts. Perhaps more relevant, we observed a fairly flat dose–response curve to MBG and ouabain with respect to stimulation of fibroblast collagen production once the threshold for an effect was reached. We suggest that in the setting of heart failure, a condition known to have associated increases in MBG and other cardiotonic steroids, the addition of digoxin at therapeutic doses might not have a detectable effect. Finally, we would point out that a systemic examination of whether digoxin induces or influences cardiac fibrosis in humans has not been thoroughly investigated, although the clinical efficacy of this agent in treating congestive heart failure has been extensively examined. It is important to
note that the rate at which humans develop cardiac fibrosis seems to be considerably slower than that seen with rodents,\textsuperscript{29} which might further obfuscate whether digoxin has profibrotic effects in clinical subjects.

In summary, we observed that concentrations of MBG similar to that which develop in experimental renal failure produced increased synthesis of collagen in primary cardiac fibroblasts grown in culture in a manner dependent on signaling through an Na/K–ATPase–Src–EGFR–reactive oxygen species signaling cascade.\textsuperscript{13, 14, 30} Should these data be confirmed in humans, this insight may provide useful therapeutic targets in clinical uremic cardiomyopathy.

\textbf{Perspectives}

Cardiac fibrosis is an important component of cardiac diseases seen in a variety of disease states. Our data in the experimental renal failure model suggest that cardiotonic steroids, such as MBG, may contribute in a very substantial role in the cardiac fibrosis seen in this setting. Because increases in MBG are likely to accompany a variety of volume expansion states, the implications of our observations may extend to other situations complicated by cardiac fibrosis.
Acknowledgment

We thank Carol Woods for her excellent secretarial assistance.

Sources of Funding

Portions of this study were supported by the American Heart Association (D.J.K., fellowship award from the Ohio Valley Affiliate) and the National Institutes of Health (HL67963). This work has also been supported in part by the Intramural Research Program, National Institute on Aging, National Institutes of Health.

Disclosures

None
References


Na+/K+-ATPase and the epidermal growth factor receptor relays the signal from ouabain 

hypertrophic signaling within caveolae membrane subdomains triggered by ouabain and 


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Marinobufagenin, an endogenous ligand of alpha-1 sodium pump, is a marker of 

2238: a new antihypertensive compound that modulates renal Na-K pump function 

Mammalian bufadienolide is synthesized from cholesterol in the adrenal cortex by a


<table>
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<th>Measurements</th>
<th>Sham</th>
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<th>MBG</th>
<th>PNx-IM</th>
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<td>367±9</td>
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<td>End systolic volume, μL</td>
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Analyses were performed 4 weeks after sham operation (Sham, n=20), partial nephrectomy (PNx, n=20), MBG infusion (MBG, n=20), or immunization against MBG prior to partial nephrectomy (PNx-IM, n=20). Results reported as mean±SEM.

*P<0.05 vs Sham; †P<0.01 vs Sham, comparing PNX-IM and PNx-Adx to PNx; ‡P<0.05; and §P<0.01.
**Figure Legends**

**Figure 1.** a, Representative pressure–volume loops obtained during vena cava occlusion from rats subjected to sham surgery (Sham), PNx, MBG infusion (MBG), and PNx after immunization against an MBG–albumin conjugate (PNx-IM). Quantitative data reported in the Table. Regression lines fit to the end systolic pressure volume relationship (ESPVR, dotted line) and the end diastolic pressure volume relationship (EDPVR, solid line). b, ventricular cross-sectional areas determined from trichrome stains of tissue obtained from Sham, PNx, MBG, and PNx-IM animals (each group: N=8 animals, ≈100 measurements averaged to determine mean for each animal; data shown as the group mean±SEM using N=8). Representative Immunohistochemistry images of cardiac tissues stained for (c) collagen-1 and (d) α smooth muscle actin (αSMA). Counterstain for both c and d was hematoxlyn. Western blot and corresponding densitometric analysis for (e) procollagen-1 and (f) αSMA. Note that both collagen-1 and αSMA staining are much more intense in the PNx and MBG groups compared with Sham, whereas the PNx-IM staining is similar to Sham. Similarly, procollagen and αSMA expression are substantially higher in PNx and MBG animals compared with Sham, whereas immunization against MBG (PNx-IM) attenuated the changes seen with PNx. Data for e and f are derived from N=6 experiments in each group and shown as mean±SEM. Sham refers to hearts isolated from control animals, PNx refers to PNx, MBG refers to MBG supplemented, and PNx-IM refers to animals immunized against MBG before PNx surgery. *P<0.05, **P<0.01 vs Sham, #P<0.01 vs PNx.
Figure 2. Representative Western blot for and quantitative densitometric data shown for (a) TGF-β1, (b) Smad 2/3, (c) Smad 4, and (d) pSmad 2/3. Data derived from N=6 experiments in each group and shown as mean±SEM. Note similar expression of these proteins in all of the 4 experimental groups.

Figure 3. Representative Western blot for procollagen and quantitative densitometric data obtained in response to different doses of (a) MBG (all N=10), (b) MBG 10 nM contrasted with different doses of ouabain (0.1 to 100 nM) and digoxin (10 nM; all N=8). c, ouabain sensitive 86Rb uptake as a function of MBG and ouabain concentration (N=4 at each concentration for both MBG and ouabain; data expressed as fraction of control). d, relative proline incorporation in the supernatant and matrix, both with and without collagenase digestion (total). Each group (controls and different doses of MBG) contains N=7 replicants. The difference between the total and after collagenase digestion is reported as collagen. The matrix is the sample that was obtained after removing supernatant and scraping the culture dish. e, mRNA for collagen 1 in MBG-treated (10 nM; N=8) or control (N=8) fibroblasts. f, procollagen stability after cycloheximide treatment. Time 0 is 1 hour after incubation with cycloheximide (20 μg/mL). Densitometric data displayed on log10 scale. Least square regression line fit to control (CTL) and MBG data. Bars on quantitative graphs represent the mean±SEM. *P<0.05 and **P<0.01 vs control.
**Figure 4.** a, Effects of PP2 (1 μmol/L), herbimycin (1 μmol/L), AG1478 (250 nM), and N-acetyl cysteine (2.5 mmol/L) on MBG (10 nM) stimulation of procollagen expression. The PP2, herbimycin, AG1478, and N-acetyl cysteine were administered from 2 hours before the addition of MBG and continued throughout the 24 hours of MBG incubation (total of 26 hours). Each bar represents the mean±SEM of N=8 experiments. b, effects of PP2 (1 μmol/L) and N-acetyl cysteine (2.5 mmol/L) on MBG (10 nM)-stimulated proline incorporation into collagen. Again, the PP2 and N-acetyl cysteine were added 2 hours before exposure to MBG. Each bar represents the mean±SEM of N=5 experiments. c, effects of MBG 10 nM on procollagen content in SYF and SYF+ cells. Representative Western blots are shown above quantitative data. SYF blots loaded with 15 μg of protein and SYF+ blots loaded with 10 μg of protein. Each bar represents the mean±SEM of N=6 determinations in each group. **P<0.01 vs control.

**Figure 5.** a, Effect of 24 hours of MBG (1 and 10 nM) on TGF-β, Smad 2/3, Smad 4, and pSmad 2/3 expression determined by Western blot. b and c, effects of 24 hours of MBG (10 nM), TGF-β (5 ng/mL), and the TGF-β receptor antagonist SB431542 (100 μmol/L) on procollagen-1 expression (Western blot) and radiolabeled proline incorporation, respectively. SB431542 was added 2 hours before exposure to either TGF-β or MBG (total of 26-hour exposure). Each bar represents the mean±SEM of 6 to 8 determinations. *P<0.05, **P<0.01 vs control.
Figure 2a

![Bar graph showing TGF β1 expression across different groups: Sham, PNx, MBG, PNx-IM.]

Figure 2b

![Bar graph showing Smad-2/3 expression across different groups: Sham, PNx, MBG, PNx-IM.]

51
Figure 2c

![Graph showing Smad-4 expression in different conditions]

Figure 2d

![Graph showing p-Smad2/3 expression in different conditions]
Figure 3c

![Graph showing Rb Uptake vs Molar Concentration](image)

Figure 3d

![Graph showing Relative Rationale Incorporation vs [MBG] nM](image)
Figure 4c

Figure 5a
**Figure Legends (Supplements)**

**Figure 1:** Representative immunofluorescence (top row) and corresponding trans-illuminated phase contrast microscopic images (bottom row) obtained from primary cardiac fibroblasts grown in culture. Note immunostaining to vimentin, a marker of fibroblasts is quite bright whereas staining against von Willebrand factor (a marker of endothelial cells), tropomyosin and myosin (cardiac myocyte markers) are absent.

**Figure 2:** Representative trichrome stained microscopic sections from rats subjected to partial nephrectomy (PNx, N=8) and partial nephrectomy and coincident adrenalectomy (PNx-ADx, N=11) are shown in panel (a). Panel (b) shows representative immunohistochemistry staining against Collagen-1 (top figures) and α smooth muscle actin (α SMC) (bottom figures) in samples from the same groups (both N=6). Note that PNx-ADx resulted in marked attenuation of trichrome or immunohistochemical staining against collagen-1 and αSMA compared with PNx alone.

**Figure 3:** Panel (a) shows representative fluorescence images from fibroblasts loaded with CM-DCF. Panel (b) shows quantitative data normalized to the basal level of fluorescence obtained in control cells at the beginning of experiments. In all experiments, loading of CM-DCF was performed in a standard manner, and at the beginning of each set of experiments, camera and microscope settings were set for the remainder of the day. Individual data points shown (control red circles, MBG 10nM green squares) with larger
symbols reflecting group mean ± SEM. Regression lines fit to mean data demonstrated statistically significant differences in both slope and intercept (both p< 0.01).
Supplement Figure 1

Supplement Figure 2a
Supplement Figure 2b

Collagen-1

PNx  PNx-ADx

αSMA

PNx  PNx-ADx

Supplement Figure 3a

Control

Baseline  10 Min

MBG 10nM
Supplement Figure 3b

![Graph showing fluorescence over time]

- **Control**
  - Slope = $3.4 \times 10^{-4}$

- **MBG**
  - Slope = $2.3 \times 10^{-4}$

Fluorescence (arb. units) vs. Time (seconds)
Partial Nephrectomy as a Model for Uremic Cardiomyopathy in the Mouse

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**Short Title:** Mouse model for uremic cardiomyopathy

**Key Words:** renal failure; TGF-β; cardiotonic steroids; reactive oxygen species; fibrosis
ABSTRACT

**Background:** Because of the plethora of genetic manipulations available in the mouse, we performed a partial nephrectomy in the mouse and examined whether the phenotypical features of uremic cardiomyopathy described in humans and rats were also present in the murine model.

**Methods:** A 5/6 nephrectomy was performed using a combination of electrocautery to decrease renal mass on the left kidney and right surgical nephrectomy.

**Results:** This procedure produced substantial and persistent hypertension as well as increases in circulating concentrations of marinobufagenin. Invasive physiological measurements of cardiac function demonstrated that the 5/6 nephrectomy resulted in impairment of both active and passive left ventricular relaxation at 4 wk whereas tissue Doppler imaging detected changes in diastolic function after 6 wk. Morphologically, hearts demonstrated enlargement and progressive fibrosis, and biochemical measurements demonstrated downregulation of the sarcoplasmic reticulum calcium ATPase as well as increases in collagen-1, fibronectin, and vimentin expression.

**Conclusion:** Our results suggest that partial nephrectomy in the mouse establishes a model of uremic cardiomyopathy which shares phenotypical features with the rat model as well as patients with chronic renal failure.
INTRODUCTION

Cardiac disease is directly responsible for the extremely high morbidity and mortality seen in patients with end-stage renal disease (ESRD) (12). Clinically, this cardiac disease of renal failure, also called uremic cardiomyopathy, is characterized by left ventricular hypertrophy and diastolic dysfunction. On this background, we have previously demonstrated that the cardiotonic steroid marinobufagenin (MBG), signaling through the Na-K-ATPase, is responsible for many of the features of experimental uremic cardiomyopathy induced by partial nephrectomy in the Sprague-Dawley rat (6). Specifically, we have noted that partial nephrectomy in the rat is accompanied by substantial elevations in blood pressure, cardiac hypertrophy, impaired left ventricular relaxation, downregulation of the sarcoplasmic reticulum ATPase (SERCA) and cardiac fibrosis. Except for the blood pressure elevation, immunization against MBG prevents all of these abnormalities (2, 6).

Although the rat is an extremely useful model to study the cardiomyopathy of renal failure (5, 6), there are many genetic manipulations which are currently available in the mouse as well as greater ease of making additional genetic manipulations in a murine system. Therefore, we performed the following studies to test the feasibility of studying experimental uremic cardiomyopathy in the mouse.
METHODS

All animal experimentation described in the manuscript was conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* using protocols approved by the University of Toledo Institutional Animal Care and Use Committee. We performed a partial nephrectomy (PNx) in male CD1 mice, weighing between 25 and 27 g. PNx was performed by selective cauterization (Bovie high-temperature fine tip cautery, Aaron Medical, St. Petersburg, FL) of the entire upper and lower poles of the left kidney, leaving a 2-mm intact segment around the hilum (Fig. 1) as described by Gagnon and Duguid (3). This was followed by removal of the right kidney at the same time. Following PNx or sham surgery, conscious blood pressure was monitored weekly using the tail-cuff method (6). In a substudy to address the role of blood pressure, \( n = 13 \) mice were given antihypertensive therapy consisting of hydralazine (80 mg/l), reserpine (5 mg/l), and hydrochlorothiazide (30 mg/l) added to the drinking water (15).

Some mice were subsequently anesthetized with pentobarbital sodium (50 mg/kg ip) and studied with Doppler imaging at 4 and 6 wk following surgery or instrumented with a Millar 1.4-Fr catheter at either 4, 6, and 8 wk for measurement of ventricular hemodynamics [e.g., \( \tau \) value, slope of regression line fit to end-diastolic pressure vs. end-diastolic volume generated by inferior vena cava occlusions (EDPVR)] as we have previously reported in the rat (2).

A Sonos 5500 system using a 14-MHz linear transducer (15-6L, Philips Medical Systems, Bothell, WA) was used to perform Doppler imaging studies as described by
other workers (7, 13, 14). Diastolic function was assessed by examination of the early (Ea) and late or atrial (Aa) velocity waves on the tissue Doppler imaging (TDI) studies as well as the early (E) and atrial (A) wave on the flow Doppler studies. For the ventricular catheter studies, diastolic function was assessed by measuring the time constant for isovolumic relaxation (τ) to assess active relaxation and the EDPVR to assess passive relaxation. Higher values of τ and EDPVR imply impaired active and passive relaxation, respectively (2, 8, 10, 18).

Blood was sampled for measurement of plasma MBG concentration ([MBG]), and the animal’s heart was removed and studied for weight, histology (trichrome staining and morphometric analysis), and biochemical analysis as we have previously reported in the rat (2, 6). Plasma [MBG] was determined following extraction on a C18 column using an immunoassay employing DELPHIA as previously described (6).

Western blot analysis was performed on protein isolated from tissue homogenates as described previously (5). Data are presented as means ± SE.

Data obtained were first tested for normality. If the data did not pass the normality test, the Tukey test (for multiple groups) or the Mann-Whitney Rank Sum test was used to compare the data. If the data did pass the normality test, parametric comparisons were performed. If more than two groups were compared, one-way analysis of variance was performed before comparison of individual groups with the unpaired Student’s t-test with Bonferroni’s correction for multiple comparisons (16). Statistical analysis was performed using SPSS software.
RESULTS

Induction of PNx resulted in rapid and sustained increases in blood pressure (Fig. 1A). Plasma [MBG] was noted to be increased about two fold 4, 6, and 8 wk following PNx compared with sham surgery ($P < 0.05$ at each time; see Table 2). For examination of left ventricular function, PNx resulted in increases in systolic function as assessed by ejection fraction, $dp/dt$, and the slope of the end-systolic pressure-volume relationship (ESPVR) following inferior vena cava constriction (Table 1). Regarding diastolic function, both active relaxation assessed by the time constant for isovolumic relaxation ($\tau$) and passive relaxation assessed by the slope of the EDPVR were noted to be impaired by PNx (representative pressure-volume loops in Fig. 1B, data in Table 1). TDI measurements also noted trends of decreasing Ea and Aa velocities with time, but only the Aa velocity was significantly reduced at 6 wk. Marked ventricular hypertrophy was noted at 4, 6, and 8 wk following PNx as demonstrated by increases in the heart weight-to-body weight ratio (see Table 3). Interestingly, end-diastolic and end-systolic volumes were noted to be markedly reduced at 4 wk after PNx, using the Millar catheter system, whereas these measurements appeared to increase at 6 and 8 wk. We suspect that this “normalization” of these volumes may actually represent a conversion from concentric to eccentric hypertrophy and a worsening of the cardiomyopathy (9), but additional studies with longer time periods of observation will be necessary to confirm this.

Time control hearts did not demonstrate any increase in fibrosis by morphology or any cardiac hypertrophy or increase in myocyte cross-sectional area during the course of the study. Similarly, protein expression data were similar at the three time points studied.
In contrast, PNx induced activation of sarcoma viral oncogene homolog (Src) and ERK at 4, 6, and 8 wk, signal transduction steps which we have previously shown to be important in signaling through the Na-K-ATPase (6). Decreases in both $\alpha_1$- and $\alpha_2$-Na-K-ATPase isoform expressions were also noted as well as decreases in SERCA2a (Table 2). Trichrome staining of histological sections demonstrated increases in myocyte cross-sectional area as well as increased fibrosis (representative images in Fig. 2, quantitative data in Table 3). Increases in procollagen and vimentin expression were noted as well (Data not shown).

Interestingly, the cardiac changes induced by PNx were not measurably altered by reductions in blood pressure achieved by the antihypertensive agents. The addition of these agents resulted in substantial reductions in systolic BP at 4 wk (126 ± 2 mmHg, $P < 0.01$ vs. PNx alone) but did not substantially affect the cardiac alterations induced as assessed by the heart weight-to-body weight ratio (4.6 ± 0.1 × 10$^{-3}$, $P = $ not significant vs. PNx alone) or the amount of fibrosis on trichrome staining (14 ± 3%, $P = $ not significant vs. PNx alone) assessed after 4 wk.
DISCUSSION

Our laboratory has been interested in the role that cardiotonic steroids play in the pathogenesis of uremic cardiomyopathy. Specifically, we have noted that a number of biochemical, physiological, and morphological changes occur with PNx in the rat and that MBG, signaling through the Na-K-ATPase, appears to be important in this process. However, our studies are still inconclusive without the capacity to examine the actual signaling process in vivo. We performed the studies in this report in the mouse to establish that the same phenotypical changes followed partial nephrectomy so that we might use a murine model for further mechanistic studies.

Our data demonstrated that the CD1 mouse responded quite similarly to PNx as we previously reported with Sprague-Dawley rats (2, 5, 6). Specifically, we were able to demonstrate sustained increases in conscious blood pressure and plasma [MBG], cardiac hypertrophy, evidence for impaired active and passive relaxation and progressive cardiac fibrosis following PNx in the male CD1 mouse. Although TDI and flow Doppler assessments were not as sensitive as the Millar pressure catheter to changes in left ventricular relaxation, the results of the TDI studies further demonstrated left ventricular diastolic dysfunction consistent with uremic cardiomyopathy. This apparent insensitivity of the echocardiographic measurements was likely a consequence of suboptimal performance rather than an inherent limitation of this technique. In general, the physiological, morphological, and biochemical alterations with PNx were similar to that which we have reported in the male Sprague-Dawley rat, where suprarenal aortic constriction was used as a control for hypertensive changes (5). Interestingly, the CD1
mouse also develops severe hypertension with suprarenal aortic constriction but very little cardiac hypertrophy or fibrosis over a similar time course to what we employed in our current study (4, 17). Moreover, in the current study, “triple” antihypertensive therapy (15) substantially lowered blood pressure toward normal, but it did not attenuate the cardiac hypertrophy or fibrosis induced by PNx. Thus the cardiac changes seen in this model appear to be more dependent on the uremic milieu rather than elevations in blood pressure alone. This report demonstrates the feasibility of pursuing detailed studies of the molecular mechanisms utilizing knockout and “knockin” models that have already been established in the mouse. For example, the role of caveolin-1 can be studied using the caveolin-1 knockout mouse developed by Razani and colleagues (11). Alternatively, the role of the Na-K-ATPase as a receptor in this process can be studied using $\alpha_1$-Na-KATPase knockout (heterozygote), $\alpha_2$-resistant, and $\alpha_1$-sensitized mice created by Lingrell and coworkers (1, 19). While these future studies are both interesting and potentially important, we believe that this brief technical report demonstrates the feasibility of using the mouse model for these studies as well as other investigations into the pathogenesis of uremic cardiomyopathy.
ACKNOWLEDGMENTS

The authors thank Carol Woods for excellent secretarial assistance. Some of these data were presented in abstract form at the 2006 American Society of Nephrology Meeting, November 14–19, San Diego, CA.

GRANTS

Portions of this study were supported by the American Heart Association (Fellowship award from the Ohio Valley Affiliate to D. J. Kennedy) and National Institutes of Health (NIH) Grant HL-67963. This work has also been supported by the Intramural Research Program, National Institute on Aging, NIH.
REFERENCES


Table I. Effects of PNx on various functional parameters.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Sham</th>
<th>4 wk</th>
<th>6 wk</th>
<th>8 wk</th>
</tr>
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<tbody>
<tr>
<td>Doppler imaging</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ea wave (TDI endocardium), m/s</td>
<td>0.042±0.002</td>
<td>0.041±0.004</td>
<td>0.039±0.003</td>
<td></td>
</tr>
<tr>
<td>Aa wave (TDI endocardium), m/s</td>
<td>0.039±0.003</td>
<td>0.032±0.006</td>
<td>0.030±0.001*</td>
<td></td>
</tr>
<tr>
<td>S wave (TDI endocardium), m/s</td>
<td>0.038±0.002</td>
<td>0.040±0.002</td>
<td>0.045±0.004</td>
<td></td>
</tr>
<tr>
<td>E wave (Mitral inflow), m/s</td>
<td>1.11±0.07</td>
<td>1.08±0.06</td>
<td>1.06±0.09</td>
<td></td>
</tr>
<tr>
<td>A wave (Mitral inflow), m/s</td>
<td>0.69±0.05</td>
<td>0.64±0.02</td>
<td>0.61±0.07</td>
<td></td>
</tr>
<tr>
<td>Left ventricular catheter</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>End-systolic volume, μl</td>
<td>6.7±0.3</td>
<td>3.7±0.2†</td>
<td>4.1±0.3†</td>
<td>4.2±0.3†</td>
</tr>
<tr>
<td>End-diastolic volume, μl</td>
<td>18.5±0.3</td>
<td>12.9±1.5†</td>
<td>16.2±1.2*</td>
<td>17.6±0.8</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>73±1</td>
<td>80±1†</td>
<td>82±1†</td>
<td>82±2†</td>
</tr>
<tr>
<td></td>
<td>4 wk</td>
<td>6 wk</td>
<td>8 wk</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td><strong>τ, ms</strong></td>
<td>7.5±0.3</td>
<td>10.5±0.4†</td>
<td>11.0±0.8†</td>
<td>10.5±0.4†</td>
</tr>
<tr>
<td><strong>EDPVR, (mmHg/μl) × 10^4</strong></td>
<td>310±38</td>
<td>563±75*</td>
<td>765±64†</td>
<td>677±76†</td>
</tr>
</tbody>
</table>

Values are means ± SE. TDI, tissue doppler imaging; PNx, partial nephrectomy; ND, not done; τ, time constant for isovolumic relaxation; EDPVR, end-diastolic pressure vs. end-diastolic volume generated by inferior vena cava occlusions. Studies were performed 4 and 6 wk, and 4, 6, and 8 wk after sham or PNx surgery for the echocardiography and ventricular catheterization, respectively. Sham surgery mice were found to have virtually identical measurements at the different time points for both Doppler imaging [4 (n = 6) and 6 wk (n = 7)] and ventricular catheterization studies [4 (n = 12), 6 (n = 8), and 8 wk (n = 8)]. To simplify the table, these sham surgery data have been combined. *P < 0.05 vs. sham. †P < 0.01 vs. sham.
Table II. Effect of PNx on various biochemical measurements.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Sham ($n = 28$)</th>
<th>4 wk ($n = 12$)</th>
<th>6 wk ($n = 8$)</th>
<th>8 wk ($n = 8$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma [MBG], pmol/l</td>
<td>143±22</td>
<td>304±55*</td>
<td>331±79*</td>
<td>343±69*</td>
</tr>
<tr>
<td>Plasma malondialdehyde μM</td>
<td>2.6±0.1</td>
<td>2.8±0.1</td>
<td>2.8±0.2</td>
<td>3.2±0.1*</td>
</tr>
<tr>
<td>Western blot analyses</td>
<td>$n = 18$</td>
<td>$n = 6$</td>
<td>$n = 6$</td>
<td>$n = 6$</td>
</tr>
<tr>
<td>pSrc/Src, fraction of control</td>
<td>1.0±0.04</td>
<td>1.35±0.08*</td>
<td>1.22±0.10</td>
<td>1.29±0.07*</td>
</tr>
<tr>
<td>pERK/ERK, fraction of control</td>
<td>1.0±0.02</td>
<td>2.18±0.04†</td>
<td>2.74±0.08†</td>
<td>1.90±0.04†</td>
</tr>
<tr>
<td>$\alpha_1$-Na-K-ATPase, fraction of control</td>
<td>1.0±0.04</td>
<td>0.72±0.05*</td>
<td>0.71±0.05*</td>
<td>0.60±0.06†</td>
</tr>
<tr>
<td>$\alpha_2$-Na-K-ATPase, fraction of control</td>
<td>1.0±0.07</td>
<td>0.73±0.04*</td>
<td>0.74±0.05*</td>
<td>0.46±0.04†</td>
</tr>
<tr>
<td>SERCA2a, fraction of control</td>
<td>1.0±0.06</td>
<td>0.74±0.04*</td>
<td>0.64±0.04†</td>
<td>0.52±0.04†</td>
</tr>
</tbody>
</table>
Values are means ± SE. MBG, marinobufagenin concentration; SERCA, sarcoplasmic reticulum ATPase. Studies were performed 4 and 6 wk, and 4, 6, and 8 wk after sham or PNx surgery. Sham surgery mice were found to have virtually identical measurements at the different time points, and to simplify the table, the sham surgery data have been combined. *$P < 0.05$ vs. sham. †$P < 0.01$ vs. sham.
Table III. Effect of PNx on various morphological measurements.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Sham (n = 28)</th>
<th>4 wk (n = 12)</th>
<th>6 wk (n = 8)</th>
<th>8 wk (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart weight/body weight $\times 10^3$</td>
<td>3.9±0.1</td>
<td>4.7±0.2†</td>
<td>4.7±0.1†</td>
<td>4.8±0.2†</td>
</tr>
<tr>
<td>Myocyte cross-sectional area, fraction of control</td>
<td>1.00±0.10</td>
<td>1.24±0.11</td>
<td>1.38±0.07*</td>
<td>1.45±0.10†</td>
</tr>
<tr>
<td>Cardiac fibrosis, fraction of control area</td>
<td>1±1</td>
<td>12±4*</td>
<td>16±5†</td>
<td>49±10†</td>
</tr>
</tbody>
</table>

Values are means ± SE. Studies were performed 4 and 6 wk, and 4, 6, and 8 wk after sham or PNx surgery. Sham surgery mice were found to have virtually identical measurements at the different time points, and to simplify the table, the sham surgery data have been combined. *P < 0.05 vs. sham. †P < 0.01 vs. sham.
FIGURE LEGENDS

Fig. 1. Partial nephrectomy (PNx) produces hemodynamic changes consistent with fibrosis. A: blood pressure responses to PNx compared with control over the period of study. Values are means ± SE. **P < 0.01 vs. control. B: representative pressure-volume loops in control and PNx animals. C: representative Doppler imaging tracings [tissue Doppler imaging (TDI; top) and flow Doppler (mitral; bottom)] in control and PNx animals. Early (Ea), atrial (Aa), systolic (S), early (E), and atrial (A) waves annotated on control tracings.

Fig. 2. Representative Masson’s trichrome sections of left ventricular cardiac tissue in control animals as well as 4, 6, and 8 wk after partial nephrectomy (PNx).
Figure 1C

C

Control

PNx (6 wks)
Figure 2

Sham Heart

4 Week PNx Heart

6 Week PNx Heart

8 Week PNx Heart
Marinobufagenin Induces Increases in Procollagen Expression in a Process Involving Protein Kinase C and Fli-1: Implications for Uremic Cardiomyopathy

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Short Title: Cardiotonic steroids stimulate collagen production

Key Words: Cardiomyopathy; Renal Failure, TGF-β, Cardiotonic Steroids; Reactive Oxygen Species; Fibrosis
Abstract

**Background:** The cardiotonic steroid, marinobufagenin (MBG) has been implicated in the pathogenesis of cardiac fibrosis seen with experimental renal failure, and has been shown to directly stimulate cardiac fibroblasts to produce collagen. In this manuscript, we examined whether the transcription factor, Fli-1 might be involved in this process.

**Methods:** We first examined the relationship between collagen and nuclear Fli-1 expression in Fli-1 knockdown mice. Next, we used cell culture to determine whether Fli-1 expression was altered by cardiotonic steroids and how cardiotonic steroids might alter Fli-1 expression.

**Results:** The Fli-1 knockdown animals showed greater amounts of cardiac collagen expression and fibrosis compared to wild type. This phenomenon was more pronounced after 4 weeks of experimental renal failure induced by 5/6th nephrectomy. Similarly, we noted that there was a strong inverse relationship between the expressions of Fli-1 and procollagen in primary culture of rat cardiac and human dermal fibroblasts as well as a cell line derived from renal fibroblasts. Next, we observed that in response to MBG, decreases in nuclear Fli-1 accompanied increases in procollagen expression. After we transfected a Fli-1 expression vector into the renal fibroblast line, basal procollagen expression was decreased and no increases were observed in response to MBG. To further delineate the mechanisms behind this relationship, we returned to cardiac fibroblasts and observed that exposure to MBG was associated with a rapid translocation of the delta isoform of protein kinase C (PKCδ) to the nucleus that appeared to peak at
about 15 minutes as determined with confocal immunofluorescence microscopy; this apparent translocation was confirmed by Western blot. This PKC\(\delta\) translocation could be prevented by pharmacological inhibition of phospholipase C with U-73122, and MBG induced increases in procollagen expression could be prevented with the PKC\(\delta\) specific inhibitor, rottlerin but not the PKC\(\alpha\) specific inhibitor, GF109203X. Finally, immunoprecipitation of nuclear extract following MBG treatment with an antibody to Fli-1 resulted in increases in material immunoreactive to anti-phosphoserine (but not anti-phosphothreonine) and \(^{32}\)P labeled material at the molecular weight of Fli-1.

**Conclusion:** We feel these data strongly support a causal relationship between decreases in nuclear Fli-1 expression and increases in collagen production following exposure to MBG. We further suggest that MBG stimulated activation and translocation of PKC\(\delta\) which, in turn, induced phosphorylation and downregulation of the transcription factor Fli-1. Should these findings be confirmed, we speculate that this pathway may represent a therapeutic target for uremic cardiomyopathy and possibly other disorders characterized by excessive fibrosis.
Introduction

We have identified that the cardiotonic steroid, MBG, is responsible for many of the clinical features of experimental uremic cardiomyopathy, and that cardiotonic steroids, in general, directly stimulate cardiac fibroblasts to produce increased amounts of collagen (1-3). Somewhat surprisingly, we did not observe increases in TGF-β or Smad proteins either in vivo or in vitro with this process although we did find that a TGF-β antagonist, SB431542, blocked stimulation of collagen production from cardiotonic steroids (1). Because of these observations, we chose to examine other mechanisms by which cardiotonic steroids might stimulate collagen production.

Recently, the transcription factor Friend leukemia integration-1 (Fli-1), which belongs to the Ets family, has been identified as a negative regulator of collagen synthesis in dermal fibroblasts (4-6). It has also been identified that stimulation of PKC, specifically the delta isoform, can phosphorylate Fli-1 and stimulate collagen synthesis (7). Because it has been shown that cardiotonic steroids may induce increases in protein kinase C (PKC) activity through signaling through the Na/K-ATPase (8), we proposed to examine whether this pathway could explain our findings.
Materials and Methods

**Materials:** MBG (>99% pure) was isolated from the venom of *Bufa Marinus* as described previously (9). U-73122 (a PLC inhibitor) was purchased from Cayman Chemical, Ann Arbor, MI. Rottlerin, (a PKCδ inhibitor), GF109203X, (a PKCα inhibitor), and protease inhibitors were obtained from Sigma-Aldrich, St Louis, MO. Anti-type1 collagen antibody was purchased from Southern Biotech, Birmingham, AL. We used two sources of anti-Fli-1 antibody, both of which were polyclonal. One which was used in the samples derived from in vitro studies was purchased from Santa Cruz, Santa Cruz, CA whereas a rabbit anti-Fli-1 antibody which was supplied by one of the authors (DW) was used for the Western blots derived from cardiac tissue. Anti-PKC antibodies were purchased from BD Biosciences, San Jose, CA. Anti-phosphoserine and anti-phosphothreonine antibodies were obtained from Calbiochem, San Diego, CA. [³²P] orthophosphoric acid was purchased from Perkin Elmer, Waltham, MA. Normal human dermal fibroblasts were obtained from Cambrex Bioscience, Walkersville, MD, and rat renal fibroblasts were purchased from ATCC, Manassas, VI. A pSG5 plasmid expressing Fli-1 gene was employed as previously described by one of the authors (MBK) (5).

**Animal studies:** All animal experimentation described in the manuscript was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals using protocols approved by the University of Toledo Institutional Animal Care and Use (IACUC) Committee. Male mice (B6; 129SvEv-Fli-1<sup>tm1</sup>) weighing between 25-30gms were used in this study. Systolic blood pressure was measured in conscious animals using
tail-cuff method as previously described (10). The animals were then subjected to either sham surgery or partial nephrectomy (PNx) as we have also previously described (10). Following surgery, systolic blood pressure was monitored weekly until they were sacrificed. At the end of 4 weeks, mice were anesthetized with pentobarbital sodium (50mg/kg ip). Blood was collected for measurement of plasma MBG concentration. Then the animal’s heart was removed, weighed and used for histological (trichrome staining and morphometric analysis) and biochemical analysis as we have previously reported (1-3,10). Plasma MBG was determined following extraction on a C18 column using an immunoassay employing DELPHIA as previously described (1,3). Western blot analysis was performed on tissue homogenates prepared from different groups as described below.

**Cell culture:** Adult rat cardiac fibroblasts were isolated as previously described by Brilla, et al.1994 (11) with modifications as previously described by us (1). Cardiac, renal and human dermal fibroblasts were grown to confluence in DMEM with 15% FBS and starved for 18-24 hours in a medium containing 1% FBS prior to treatment. Cardiac, renal and human dermal fibroblasts were used up to passage 2, 10 and 7, respectively. Protein contents of various preparations were determined using the Bio-Rad protein assay method. U-73122, a PLC inhibitor, was used at a concentration of 10 μM in the medium. Rottlerin, a PKC inhibitor specific for PKCδ and GF109203X, a PKC inhibitor specific for PKCγ, were used at 4 μM concentrations.

**Generation of stable transfectants:** Renal fibroblasts were grown to 80% confluence and they were transfected with either pSG5 Fli-1 gene or pSG5 vector and pTargetTTM
vector carrying neomycin resistant gene (Promega, Madison, WI). The transfection was carried out at a ratio of 10 parts of pSG5-Fli-1 to 1 part of pTarget vector using a transfection reagent FuGENE 6 (Roche, Nutley, NJ) according to manufacturer’s protocol. Individual antibiotic-resistant colonies were selected and expanded in medium containing 400ug/ml G418. The cells transfected with the pSG5-Fli-1 and a pSG5 (control) genes were analyzed for the expression of procollagen determined on whole cell lysates and the nuclear expression of the Fli-1 protein.

**Western blot analysis:** Cell lysates were prepared by washing the cells twice with ice-cold phosphate-buffered saline (PBS) and incubating them on ice for 20 minutes with lysis buffer containing 10 mM Tris/HCl, pH 7.5, 0.5% Nonidet P40, protease and phosphatase inhibitors. Cell lysates were collected and vortexed for 30 s and used immediately, or stored at –80 °C. For procollagen detection, cell lysates or tissue homogenates were dissolved in loading buffer and proteins (10ug/lane) were separated by SDS/PAGE using 4-15% Tris-HCl precast ready gels (Bio-Rad, Hercules, CA). For Fli-1 detection, a large 10% gel was used and the protein loading was 120ug/lane for nuclear extract or 50ug/lane for tissue homogenates. After separation, proteins were transferred onto PVDF membranes. Membranes were blocked with 5% non-fat dry milk (Bio-Rad) in Tris-buffered saline supplemented with 0.05% Tween-20 (TBS-T) at room temperature for 2 hours and then incubated with primary antibody in blocking buffer at 4°C overnight. After washing in TBS-T, membranes were incubated for 2 hours at room temperature with horseradish peroxidase-conjugated secondary antibody in blocking buffer. After washing in TBS, membranes were developed using ECL or ECL plus (Amersham
Biosciences, Piscataway, NJ). For loading controls, tubulin or actin were probed; for both, the primary Ab was diluted 1/3000 and the secondary Ab (goat anti-mouse, Santa Cruz) were diluted 1/2000. The images captured on x-ray film were scanned using CanoScan Li DE 60 from Canon and quantified by using the Image J version 1.37V software (NIH). The quantified signals were in the linear range of our detection system.

**Immunostaining and confocal microscopy:** Cells were fixed with cold absolute methanol or 4% paraformaldehyde in PBS, permeabilized in permeabilization buffer (PBS with 0.3% Triton X-100 and 0.1% BSA) for 15 minutes, and blocked with GSDB buffer [20 mmol/L sodium phosphate, pH 7.4, with 150 mmol/L NaCl, 0.3% Triton X-100, and 16% (v/v) filtered normal goat serum] for 30 minutes at room temperature. The cells were then probed with primary antibody for 90 minutes at room temperature or overnight at 4°C (rabbit polyclonal anti PKCδ antibody, Santa Cruz Biotechnology; 1:50 dilution in GSDB). After 3 washes with permeabilization buffer, the cells were incubated with Alexa Fluor® 488-conjugated antirabbit secondary antibody for 1 hour at room temperature. After another 3 washes, the cells were counterstained with propidium iodide (Molecular Probes) to localize nuclei. Cells were then mounted using Prolong Anti-fade medium (Molecular Probes) and stored at -20°C. All images were generated with a Leica DMI RES2 confocal microscope (Wetzlar, Germany). Contrast and brightness were set to ensure that all pixels were within the linear range. Negative controls were also performed to verify the specificity of primary and secondary antibodies.
**Determination of PKCδ in the nucleus:** Cardiac and renal fibroblasts were grown to confluence and starved for 18-24hrs. Cells were treated with MBG to a final concentration of 10nM in the medium. After 15min, cells were washed with ice-cold PBS and nuclear extracts were prepared from treated and untreated cells as described by Wadman, et al. (12). PKCδ in the nuclear extract was determined by performing Western blot as described above.

**Immunoprecipitation and detection of phosphorylated Fli-1 by Western blot:** Renal fibroblasts were grown and starved as described above. The cells were treated for 15min with 10nM MBG in the medium. After washing the cells in PBS, nuclear extracts were prepared from treated and untreated cells as described by Wadman (11). Equal amounts of proteins from nuclear extracts were used for immunoprecipitation. For immunoprecipitation, nuclear proteins were incubated with the polyclonal anti-Fli-1 antibody conjugated to agarose beads at 4°C overnight. The following day the immunocomplex was sedimented and washed 3-4 times with ice-cold PBS. The washed immunocomplex was dissolved in sample buffer and the proteins were separated on a 10% gel. The separated proteins were transferred to a PVDF membrane and processed as described under Western blot analysis. To detect whether serine or threonine molecules are involved in phosphorylation of proteins, anti-phosphoserine or anti-phosphothreonine antibodies were used.
**Immunoprecipitation and detection of phosphorylated Fli-1 by autoradiography:**

For autoradiography, renal fibroblasts were grown and starved as described above. The starved cells were rinsed twice with phosphate free DMEM and then incubated at 37° C in phosphate free DMEM for 30min. The cells were replaced with fresh phosphate free DMEM containing 20uCi/ml $^{32}$P orthophosphoric acid and incubated for 2hrs. MBG was added to the cells to a final concentration 10nM and 15min later the cells were washed with ice-cold PBS. Nuclear extracts were prepared as described by Wadman (11). For immunoprecipitation, nuclear extracts with equal amounts of radioactivity from treated and untreated renal fibroblasts were incubated with the polyclonal anti-Fli-1 antibody conjugated to agarose beads at room temperature for 2hrs. The immunocomplex was washed with ice-cold PBS and dissolved in sample buffer and subjected to a SDS/PAGE on a 10% gel. The gels were dried on a gel dryer and the phosphorylated proteins were visualized by using a phosphoimager, Storm 840 from Novell and quantified by using Image Quan TLV soft ware from Novell.

**Statistical analysis:** Data presented are mean± standard error of the mean. Data obtained were first tested for normality. If the data did not pass the normality test, the Tukey test (for multiple groups) or the Mann-Whitney Rank Sum test were used to compare the data. If the data did pass the normality test, parametric comparisons were performed. If more than two groups were compared, one-way analysis of variance was performed prior to comparison of individual groups with the unpaired Student’s t-test with Bonferroni’s correction for multiple comparisons. If only two groups of normal data were compared,
the Student’s t-test was used without correction (13). Statistical analysis was performed using SPSS™ software.
Results

Relationship between Fli-1 and Collagen expression in cardiac tissues of wild type and Fli-1 knockdown mice before and after nephrectomy:

We first examined whether there was a relationship between collagen production and Fli-1 expression in cardiac tissues. We determined the expression of Fli-1 and collagen in the hearts obtained from wild and Fli-1 knockdown (KD) mice using Western blot. We observed that the hearts of the KD mice expressed approximately 50% of the Fli-1 of WT hearts (figure 1a). Next, we performed either sham or partial nephrectomy (PNx) surgery on these animals and conscious systolic blood pressure was monitored before and after surgery as the renal failure progresses. We found that systolic blood pressure was increased in WT as well as KD animals following PNx compared to sham surgery. Interestingly, the increase in systolic BP was greater in the KD animals (figure 1b) compared to WT animals. When we examined the heart size in these animals, the heart size was increased following PNx in both WT and KD animals, but the increase in heart size was greater in the KD animals (figure 1c). Next, we examined the histology of the hearts using trichrome staining; we noted that there was a greater degree of fibrosis determined by the amount of blue staining in both WT and KD animals following PNx, but the amount of blue staining was greater in the KD animals compared to the WT. (figures 1d and 1e). Of interest, cardiac myocyte cross sectional areas were not different between KD and WT animals either at baseline or after PNx, but the cross sectional areas of both KD and WT increased following PNx compared to sham surgery (figure 1f). We
also measured collagen expression in cardiac tissues from these animals. We found that collagen expression was high in KD compared to WT animals. This difference was more pronounced following PNx (figure 1g). This finding was consistent with the histological observations described above.

**Relationship between procollagen and nuclear Fli-1 expression in fibroblasts from different tissues:**

We initially examined the basal expression levels of nuclear Fli-1 and procollagen determined on whole cell lysates from fibroblasts isolated from human skin, rat heart and kidneys and grown to confluence. We noticed indeed that the procollagen production from cardiac fibroblasts was greater at baseline than that seen with the renal fibroblasts which, in turn, were greater than that seen with the human dermal fibroblasts. Interestingly, basal nuclear Fli-1 expression appeared to be strongly but inversely correlated with the basal expression of procollagen (figure 2a). Next, we examined the effect of MBG on procollagen and Fli-1 expression in all three fibroblasts. We noted that MBG increased procollagen expression in the renal and dermal fibroblasts, as well as in the cardiac fibroblasts, as we previously reported (1), (figure 2b). The threshold for significant increases in procollagen expression was at 100 pM in the renal and dermal fibroblasts, whereas it was 1 nM for the cardiac fibroblasts. Other cardiac glycosides (ouabain and digoxin) also induced increases in procollagen expression in all three cell types (data not shown) as we had previously reported in the cardiac fibroblasts (1). MBG
administered at a 10 nM concentration resulted in marked and similar decreases in nuclear Fli-1 expression in all fibroblasts studied (figure 2c).

**Decreases in nuclear Fli-1 expression are necessary for MBG induced increases in procollagen expression in a renal fibroblast line:**

To further examine the relationship between Fli-1 expression and MBG induced increases in procollagen production, we stably transfected the renal fibroblasts with a Fli-1 expression vector coupled to an SV40 promoter. We found that the renal fibroblasts transfected with Fli-1 expression vector showed marked increase in nuclear Fli-1 expression as expected. At the same time, the basal expression level of procollagen was significantly reduced. Furthermore, when these transfected cells were exposed to MBG, MBG did not produce an increase in procollagen expression. On the other hand, MBG produced a significant increase in procollagen expression as well as decrease in nuclear Fli-1 expression in control renal fibroblasts (transfected with an empty vector, figure 3).

**MBG induces translocation of PKCδ into the nucleus:**

In this set of experiments, we first examined the expression of various PKC isoforms in cardiac fibroblasts. We found that the α, γ, δ, ε and λ PKC isoforms could all be detected with Western blot. However, following MBG administration, only the PKCδ expression was increased in the nuclear fraction. Based on the work of Jinnin and colleagues (7), we examined the effect of rottlerin, a specific inhibitor of PKCδ on the MBG induced expression of procollagen. We found that addition of rottlerin (4 μM),
resulted in marked reductions in basal procollagen expression and prevented MBG
induced increases in procollagen expression (figure 4c). In contrast, administration of
GF109203X which is specific for PKCα did not alter the change in procollagen
expression following MBG treatment for 24 hours (relative expression to control = 1.9 ±
0.2, N=3).

We next used confocal immunofluorescence microscopy technique to follow the
time course of what we presumed was PKCδ translocation to the nucleus. Confocal
immunofluorescence microscopy experiments showed that that the nuclear PKCδ
expression peaked at about 15 minutes and slowly decreased over the next 24 hours of
exposure to MBG (figure 4a). An identical pattern was noted with renal fibroblasts (N=5,
data not shown). The finding of confocal experiment was supported by Western blot
experiment where we found a significant increase in cardiac nuclear PKCδ expression at
15 minutes after exposure to 10 nM MBG. This increase was blocked by co-incubation
with the PLC inhibitor, U-73122 (figure 4b). Again, a virtually identical pattern was seen
with the renal fibroblasts (N=4, data not shown).

Our next step was to examine whether the translocation of PKCδ into the nucleus
causes phosphorylation of Fli-1. To test this, cells exposed to 10nM MBG for 15min and
nuclear extracts were prepared. To enrich Fli-1, nuclear extract was incubated with
polyclonal anti-Fli-1 antibody. The resulting immunocomplex was separated on a 10%
gel and probed for phosphorylated proteins, particularly Fli-1 using an anti-
phosphoserine Ab. We found a band with greater intensity corresponding to the
molecular mass of Fli-1 (figure 5a). When the experiment was repeated in the presence
of rottlerin or U-73122 (both N=3), the intensity of the 50 kDa band was the same or less than that seen with untreated cells (blot densities of 0.7 ± 0.3 and 0.7 ± 0.2 with rottlerin or U-73122 alone, respectively and 0.6 ± 0.3 and 0.7 ± 0.2 when rottlerin or U-73122 was combined with MBG, respectively (data expressed relative to control, both N=3). When the immunocomplex was probed after separation of proteins with an anti-threonine Ab we did not see any increase in intensity of the band with 50kDa mass following MBG treatment (blot density of 0.9 ± 0.2 relative to control, N=3).

To further demonstrate that MBG induced decreases in Fli-1 expression through PKCδ mediated phosphorylation, we labeled the cells with ³²P and prepared nuclear extracts. To enrich Fli-1, nuclear extracts were immunoprecipitated with polyclonal anti-Fli-1 antibody and the immunocomplex was subjected to gel electrophoresis. ³²P labeled proteins present on the gel were quantified using a phosphoimager. We observed an increase in ³²P labeled protein at the level corresponding to molecular mass of Fli-1 (figure 5b); this was attenuated to be comparable to that seen in control samples by co-administration of rottlerin (blot density of 0.7 ± 0.2 with rottlerin alone and 0.6 ± 0.3 with rottlerin + MBG, data expressed relative to control, N=3).
Discussion

Uremic cardiomyopathy is characterized by diastolic dysfunction and left ventricular hypertrophy. Cardiac disease is responsible for the high mortality seen in patients suffering from kidney diseases (14). Our group and others have observed that the cardiotonic steroid marinobufagenin (MBG), signaling through the Na/K-ATPase, is directly responsible for many features of experimental uremic cardiomyopathy. MBG directly induces cardiac fibroblasts to produce collagen, thus producing much of the cardiac fibrosis seen with experimental renal failure.(1,3).

Fli-1 (Friend leukemia virus integration 1) is a member of the Ets oncongene family of proteins (15-19) and normally competes with Ets-1 in a Sp-1 dependent to balance between stimulating and repressing the Col1a2 promoter. This transcription factor has been clearly shown to play a role in dermal fibrosis, and a direct inhibitory effect on collagen -1 synthesis has been demonstrated (4). In the current study, we extended this observation to an animal model and used Fli-1 knockdown mice looking for cardiac fibrosis. First, we demonstrated that the viable heterozygotes (Fli-1\(^{+/-}\)) expressed about 50% of cardiac Fli-1 compared to wild type mice. These heterozygotes also had greater degrees of cardiac fibrosis and cardiac collagen expression compared to wild type. This difference in the cardiac fibrosis and cardiac collagen expression between heterozygotes and wild type was more pronounced in PNx animals’ expression both with and without antecedent PNx surgery than wild type mice, thus supporting the concept that
the downregulation of nuclear Fli-1 expression induced by cardiotonic steroids plays a significant role in the cardiac fibrosis seen in experimental renal failure (1,10).

Based on these in vivo results, we examined the relevance to different types of fibroblasts in particular cardiac, renal, and dermal fibroblasts. First, we demonstrated an inverse relationship between basal nuclear Fli-1 expression and collagen production in the three types of fibroblasts. We further demonstrated that MBG induced corresponding decreases in nuclear Fli-1 expression with increases in procollagen expression in each of these fibroblasts, and we confirmed the importance of Fli-1 with an overexpression transfection in the renal fibroblast cell line. These transfected cells showed higher basal levels of nuclear Fli-1 expression and lower basal levels of procollagen expression and did not show any significant increase in procollagen expression in response to MBG.

To further explore the signaling pathways leading to this observation, we first looked at the protein kinase C (PKC) family of proteins that mediates the specific activation of a variety of transcription factors. Specifically, Jinnin and colleagues have shown that PKC-delta stimulation can phosphorylate Fli-1 and increase collagen synthesis (7). Moreover, Watson, et al., suggested that a balance between protein kinase and protein phosphatase regulate the phosphorylation status and effectiveness of Fli-1 in inhibiting collagen-1 synthesis (20). We observed that MBG induced translocation of PKCδ into the nucleus is a PLC dependent process, a finding consistent with what Kometiani and coworkers demonstrated for ouabain induced activation of the Na/K-ATPase signal cascade (8). U-73122 completely prevented any increase in nuclear PKCδ whereas rottlerin prevented MBG induced increases in procollagen expression supporting
this concept. We further determined that it is PKCδ which phosphorylates Fli-1 as assessed by immunoprecipitation, immunoblotting and \(^{32}\)P labeling.

Taken together with existing literature, our findings strongly implicate the transcription factor, Fli-1, in the progressive cardiac fibrosis seen with experimental uremia as indicated in figure 6. If these data are confirmed by further studies, we would suggest that the signal cascade that has been elucidated may serve as a therapeutic target for clinical uremic cardiomyopathy.

**Acknowledgements**

We would like to thank Ms. Carol Woods for her excellent secretarial assistance. Portions of this work were supported by grants from the NIH (RO1-HL67963 and P01-CA78582).


Figure Legends

Figure 1. Characterization of cardiac fibrosis in Fli-1 knockdown animals and their response to partial nephrectomy.

Panel a shows basal Fli-1 expression determined with Western blot (representative blot at top, quantitative data at bottom) in cardiac tissue obtained from N=5 wild type (WT) and Fli-1 knockdown (KD) mice. Panel b shows conscious systolic blood pressure in WT animals exposed to sham (N=6) and partial nephrectomy (PNx, N=8) surgery as well as KD mice exposed to sham (N=6) and PNx (N=9) surgery determined at baseline, 1, 2, 3 and 4 weeks. Panel c shows the heart weight normalized for body weight in these animals at 4 weeks. Panel d shows representative histology (trichrome stain) and panel e shows the quantification of the fibrosis and panel f shows the measurement of myocyte cross sectional area from the hearts of these mice. Panel g shows collagen expression determined with a polyclonal antibody (representative Western blot with dimer at about 100Kd, quantified data shown below) in these animals.

* p<0.05, ** p<0.01 vs Sham-WT, # p<0.05, ## p<0.01 vs PNx-WT.

Figure 2. Procollagen and Fli-1 expression in different fibroblasts.

Panel a shows a representative Western blot for procollagen showing dimer detected with polyclonal antibody at approximately 150Kd and Fli-1 detected with a polyclonal antibody at approximately 50Kd (both shown at top) and the quantification of these measurements in each group (N=6) obtained at baseline in rat cardiac and renal and
human dermal fibroblasts grown to confluence. Regression line for procollagen expression compared to Fli-1 expression is shown with data normalized to that of cardiac fibroblasts ($r^2 = 0.98$, $p< 0.01$). Panel b shows quantitative densitometric data for procollagen (measured by Western blot as in panel a) in response to different doses of MBG for 24 hours (N=6). Panel c shows quantitative densitometric data for Fli-1 (measured by Western blot as in panel b) obtained in response to 10 nM MBG (N=6) for 24 hours. Bars on quantitative graphs represent the mean ± SEM. **P<0.01 vs. control.

**Figure 3. Effects of MBG on procollagen and Fli-1 expression in stably transfected rat renal fibroblasts.**

MBG was administered at a 10 nM for 24 hours. Bars on quantitative graphs represent the mean ± SEM of 6 determinations derived from Western blots as in Figure 1. *P<0.05 and **P<0.01 vs. control.

**Figure 4: Effect of MBG on nuclear PKCδ expression in cardiac fibroblasts.**

Panel a shows confocal immunofluorescence micrographs of cardiac fibroblasts at baseline and treated with 10 nM MBG for 15 min and 24 hrs. Images were also obtained at 5 and 30 min as well as 1 and 4 hrs but not shown. Panel b shows a nuclear PKCδ expression determined by Western blot in cells treated with 10 nM (MBG) and/or the PLC inhibitor, U-73122 (U) at 10 μM for 15 min (N=6 in each group). Panel c shows procollagen expression determined by Western blot (see figure 1) in cells treated with 10
nM MBG with or without the PKCδ inhibitor, Rottlerin 4 μM (N=6 in each group) for 24 hours. * p<0.05, ** p<0.01 vs control, # p<0.05, ## p<0.01 vs MBG.

**Figure 5: Effect of MBG on phosphorylated Fli-1 in renal fibroblasts.**

Panel a shows an immunoblot against phosphoserine in nuclear extracts immunoprecipitated with the polyclonal antibody against Fli-1; the representative blot is shown above and quantification of N=4 determinations is shown below. Panel b shows $^{32}$P autoradiograph data obtained from cells treated with $^{32}$P labeled phosphate for 2 hours and MBG for 15 minutes. The top shows a representative autoradiograph and the bottom quantification of N=5 experiments. * p<0.05 vs control.

**Figure 6: Schematic describing how cardiotonic induced sodium pump signaling may result in decreases in collagen production.**

In the presence of the cardiotonic steroid MBG, Na/K-ATPase is converted to a signal transducer which complexes with Src and the EGFR. A signal cascade is initiated which involves PLC which results in the activation of PKCδ and its translocation to the nucleus. In the nucleus, PKCδ phosphorylates Fli-1. This, in turn, leads to more rapid catabolism of Fli-1 and removal of Fli-1 inhibition on the Col1 promoter and, thus, increases in collagen expression. Data supporting this schematic summarized from references (1, 3, 21-25).
Figure 1a

![Cardiac Fli-1 Expression](chart)

- **WT**
- **KD**

Figure 1b

![Systolic Blood Pressure](chart)

- **Sham-WT**
- **Sham-KD**
- **PNx-WT**
- **PNx-KD**

**Baseline**

**Time (weeks)**

- **80**
- **100**
- **120**
- **140**
- **160**
- **180**
- **200**

**Systolic Blood Pressure (mmHg)**

- **#**
- ****
- **##**
- **##**

- **1**
- **2**
- **3**
- **4**
Figure 1c

![Heart Weight/Body Weight Ratio X 1000](chart)

- Sham-WT
- PNx-WT
- Sham-KD
- PNx-KD

Figure 1d

![Sham and PNx Images](images)

- WT
- Fli-1 KD
Figure 1g

![Procollagen expression](image)

**Procollagen Expression**

Cardiac Collagen Expression (a.u. units)

<table>
<thead>
<tr>
<th>Condition</th>
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<tbody>
<tr>
<td>Sham-WT</td>
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<tr>
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<td><strong>3</strong></td>
</tr>
<tr>
<td>PNx-KD</td>
<td>###</td>
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</table>

Figure 2a

![Procollagen and Fli-1 expression](image)

**Procollagen and Fli-1 Expression**

- **Cardiac**
- **Renal**
- **Dermal**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Procollagen</th>
<th>Fli-1</th>
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<tbody>
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<td>0.4</td>
</tr>
<tr>
<td>Renal</td>
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</tr>
<tr>
<td>Dermal</td>
<td>0.6</td>
<td>0.8</td>
</tr>
</tbody>
</table>

**Procollagen Expression**

Procollagen Expression (Relative to Cardiac)

**Fli-1 Expression**

Fli-1 Expression (Relative to Cardiac)
Figure 3

![Bar graph showing Procollagen and Fli-1 levels in Normal Cells and Transfected Cells with MBG treatment.](image)

Figure 4a

![Images showing Baseline, MBG 15 min, and MBG 24 hr conditions.](image)
Figure 4b

![Figure 4b](image)

**Figure 4c**

![Figure 4c](image)
Figure 6

Fibrosis

Procollagen 1

Procollagen 1 mRNA

Proteolysis

PKCδ

Fli-1

Col1 promoter

EGFR

Src

PLC

Caveolin

MBG
SUMMARY

Usually the term uremic cardiomyopathy refers to patients with renal failure who develop diastolic dysfunction, oxidant stress, left ventricular hypertrophy and cardiac fibrosis which are most probably related to their renal disease. Increased circulation of cardiotonic steroids such as ouabain and marinobufagenin are in part responsible for the cardiac fibrosis seen in uremic cardiomyopathy which has been demonstrated both in clinical and experimental renal failure. It is interesting to note that collagen production, a marker of fibrosis, is regulated by the transcription factor Fli-1 which is regulated by the protein kinase PKCδ in human dermal fibroblasts. Cardiotonic steroids might play a major role in the cardiac fibrosis seen in uremic cardiomyopathy and the molecular mechanism of fibrosis might involve PKCδ and Fli-1.

We have recently seen that experimental renal failure in the rat is accompanied by increases in circulating concentrations of the cardiotonic steroid, marinobufagenin (MBG), and substantial cardiac fibrosis. In the current study, we investigated the implication of MBG in the pathogenesis of the cardiac fibrosis. First we noticed that the 5/6th nephrectomy model of experimental renal failure PNx and MBG infusion induced similar but not identical phenotypic changes in hemodynamics and cardiac morphology. On the other hand, both immunization against MBG; PNx-IM and the concomitant PNx and adrenalectomy; PNx-ADx, reduced circulating MBG and substantially attenuate the
cardiac functional and morphological changes without significantly affecting blood pressure. Second, we found that MBG like his close relative ouabain induced a signal transduction cascade through the plasmalemmal Na/KATPase residing in caveolae, which results in activation of Src, transactivation of the EGFR, generation of reactive oxygen species, and, ultimately, activation of p42/44 mitogen-activated protein kinase. Ergo, we used specific antagonists for Src, EGFR and ROS and showed that these compounds blocked signal transduction through the Na/K-ATPase and therefore inhibited the fibrosis induced by MBG assessed by increases in procollagen-1 expression by cultured cardiac fibroblasts. Finally, in an attempt to determine the molecular mechanism underlying this fibrosis, we examined the expression of several proteins important in fibroblast activation such as TGF-β and the Smad proteins. Interestingly, evidence for increases in these proteins was not evident. We should point out that these results do not exclude a role for TGF-β in this process, which could have occurred at an earlier or later time of the fibrosis progression. There is a probability that other mechanisms of regulation of collagen synthesis could take place when the TGF-β pathway is interrupted.

Next, we pursued further studies of the molecular mechanisms utilizing knockout and “knockin” models that have already been established in the mouse but not yet in the rat model. Because of this we performed a partial nephrectomy in the mouse and checked whether the phenotypical features of uremic cardiomyopathy seen in humans and rats were also present in the murine model. Interestingly, CD1 mouse responded quite similarly to PNx as we previously reported with Sprague- Dawley rats. Particularly, we
demonstrated higher MBG levels in plasma and increases in blood pressure, left ventricular hypertrophy, and cardiac fibrosis following PNx in male CD1 mice. Although Doppler imaging tracing and flow Doppler assessments were not as sensitive as the Millar pressure catheter to changes in left ventricular relaxation, we were able to note evidence for impaired in active and passive relaxation evaluated by ventricular hemodynamics measurements. We should point out that the cardiac changes seen in the murine model seemed to be more dependent on the uremic milieu rather than elevations in blood pressure alone. In fact, we showed that a “triple” antihypertensive therapy considerably lowered blood pressure toward normal, but it did not attenuate the cardiac hypertrophy or fibrosis induced by PNx in the male CD1 mice. Therefore, we demonstrated that the CD1 mouse responded quite similarly to PNx as we previously reported with Sprague-Dawley rats. In our next studies we decided to use the mouse model into the pathogenesis of uremic cardiomyopathy. Specifically, we will use a knock down murine model.

Fli-1 is a transcription factor that has clearly been shown to play a role in dermal fibrosis, because of its inhibitory effect on collagen-1 synthesis. In the current study, we demonstrated an inverse relationship between basal nuclear Fli-1 expression and collagen production in cardiac and renal fibroblasts to go along with what was seen in dermal fibroblasts. Moreover, MBG induced corresponding decreases in nuclear Fli-1 expression with increases in procollagen expression in each of these fibroblasts. Next, we transfected renal fibroblasts with the Fli-1 gene; these cells showed higher basal levels of nuclear Fli-
expression and lower basal levels of procollagen expression and did not show any significant increase in procollagen expression in response to MBG. In support to the in vitro results, we look at the significance to cardiac fibrosis using a Fli-1 knockdown model. As expected, the heterozygous mice had greater degrees of cardiac fibrosis and cardiac collagen expression compared to wild type mice. This observable fact was more pronounced after 4 weeks of experimental renal failure induced by 5/6th nephrectomy in both heterozygotes and wild type. We turned back to our in vitro experiments, to look at the molecular mechanism behind this relationship. We noticed that exposure of cardiac fibroblasts to MBG, for about 15 minutes, was associated with a rapid translocation to the nucleus of a protein kinase; PKCδ. MBG induced increases in procollagen expression could be prevented by rottlerin, a PKCδ specific inhibitor, or by U-73122 a pharmacological inhibition of phospholipase C. We finally showed that PKCδ stimulation by MBG phosphorylated Fli-1 on its serine residue and thus increased collagen synthesis.

Taken together our data suggest the following pathway for cardiotonic steroids in the regulation of cardiac fibrosis seen in patients with uremic cardiomyopathy: MBG stimulates activation and translocation of the protein kinase PKCδ which phosphorylates and downregulates the transcription factor Fli-1 which will lead to increase collagen synthesis. Should these findings be well established, we speculate that this pathway may represent a therapeutic target for uremic cardiomyopathy and other fibrotic disorders.
CONCLUSIONS

1. Experimental renal failure in rats and mice is accompanied by increases in circulating concentrations of cardiotonic steroids such as marinobufagenin (MBG). This phenomenon is also observed in patients with renal diseases.

2. MBG like other cardiotonic steroids induces a signal transduction cascade through the plasmalemmal Na/KATPase residing in caveolae, which results in activation of Src, transactivation of the epidermal growth factor receptor, generation of reactive oxygen species, and activation of protein kinases.

3. In rat undergoing 5/6th nephrectomy (PNx) for 4 weeks, both immunization against MBG, and the concomitant PNx and adrenalectomy reduced circulating MBG and substantially attenuate the cardiac functional and morphological changes without significantly affecting blood pressure.

4. The same phenotypical features of uremic cardiomyopathy seen in humans and rats were also present in the murine model undertaking partial nephrectomy. The cardiac changes seen in the mice appear to depend on the uremic milieu rather than elevations in blood pressure alone.
5. Fli-1 knockdown mice had greater degrees of cardiac fibrosis and cardiac collagen expression compared to wild type mice before and after partial nephrectomy.

6. Renal fibroblasts transfected with the Fli-1 gene showed higher basal levels of nuclear Fli-1 expression and lower basal levels of procollagen expression with no significant increase in procollagen expression in response to MBG.

7. MBG stimulates activation and translocation of the protein kinase PKCδ which phosphorylates and downregulates the transcription factor Fli-1 which will lead to increase collagen synthesis.
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mellitus who have end-stage renal disease and normal epicardial coronary arteries. *Am Heart J* 147:1017-1023.


ABSTRACT

It has been recognized that patients with chronic renal failure eventually develop diastolic dysfunction, cardiac hypertrophy and systemic oxidant stress along with increases in circulating concentrations of the cardiotonic steroid, marinobufagenin (MBG) in uremic cardiomyopathy. Because of this, we performed 5/6th partial nephrectomy in rats to study experimental renal failure (PNx), MBG infusion, PNx after immunization against MBG, and concomitant PNx and adrenalectomy. We also studied 5 6th partial nephrectomy as a potential experimental renal failure model in mice. Next, we speculated a relationship between decreases Fli-1 expression and increases in collagen production following exposure to MBG. Therefore, we examined Fli-1 knockdown mice and compared it to wild type mice. Physiological measurements with a Millar catheter and immunohistochemistry were performed. In vitro studies were then pursued with cultured isolated cardiac fibroblasts, human dermal fibroblasts, as well as a cell line derived from renal fibroblasts.

First, in rats, we observed that PNx after immunization against MBG as well as concomitant PNx and adrenalectomy had similar blood pressure as PNx but less cardiac hypertrophy, diastolic dysfunction, and cardiac fibrosis. Second, in mice, the 5 6 nephrectomy resulted in impairment of both active and passive left ventricular relaxation at four weeks as well as progressive fibrosis in the heart. Third, the Fli-1 knockdown
mice showed greater amounts of cardiac collagen expression and fibrosis compared to wild type before and after 4 weeks of experimental renal failure induced by 5/6\textsuperscript{th} nephrectomy.

We realized that stimulation of cultured cardiac fibroblasts with MBG could be prevented by administration of inhibitors of tyrosine phosphorylation, Src activation, EGFR transactivation, and N-acetyl cysteine. Furthermore, in response to MBG, decreases in nuclear Fli-1 accompanied increases in procollagen expression. Finally, we observed that exposure of cardiac fibroblasts to MBG was associated with a rapid translocation of PKC\(\delta\) to the nucleus that appeared to peak at about 15 minutes as determined with confocal immunofluorescence and Western blot.

Taken together, these data suggest that MBG directly induces increases in collagen expression by fibroblasts in a process involving Fli-1 and PKC\(\delta\), and is in part responsible for the cardiac fibrosis seen with experimental renal failure.