Marinobufagenin induced uremic cardiomyopathy: the role of passive immunization, rapamycin, and CD40 signaling in the generation of renal fibrosis

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A Dissertation entitled

Marinobufagenin Induced Uremic Cardiomyopathy: The Role of Passive Immunization, Rapamycin, and CD40 Signaling in The Generation of Renal Fibrosis

By

Steven T. Haller

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Biomedical Sciences

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August 2012
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Cardiotonic steroids such as marinobufagenin (MBG) and ouabain are specific ligands for the Na/K-ATPase and represent a relatively new class of steroid hormones. Uremic cardiomyopathy is characterized by a decrease in diastolic function, left ventricular hypertrophy, oxidant stress, and both cardiac and renal fibrosis. We have shown that MBG, signaling through the Na/K-ATPase, causes many of the adverse pathological effects of experimental uremic cardiomyopathy induced by 5/6th nephrectomy (PNx) in the rat. The goal of this dissertation is to describe some of the manipulations we have performed in order to provide potential therapies for the treatment
of uremic cardiomyopathy. Specifically, we show that treatment with an anti-MBG antibody drastically reduces cardiac fibrosis in PNx animals. Treatment with rapamycin (an mTOR inhibitor) produced similar effects with the added benefit of reducing circulating MBG in these animals. In addition, we show that ischemic renal disease is accompanied with elevated levels of the platelet activation marker soluble CD40 ligand (sCD40L), and its soluble receptor, CD40, may predict outcomes in this disease state. Data in our PNx model suggests a role for proximal tubular CD40 activation contributing to the development of renal fibrosis, which may be potentiated by cardiotonic steroid signaling through the Na/K-ATPase.
Dedication

To my family who has faithfully stood by my side throughout my academic career. Your undying love and support has inspired me to be a better person. I am forever grateful for the sacrifices you have made for me along the way. Thank you for believing in me. I love you all.
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To Dr. Kennedy, My mentor, my friend, my other brother.
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Chapter 1-Literature Review and Introduction

1.1 Structure and Function of the Na/K-ATPase

The Na/K-ATPase, originally discovered by Skou (1957), is a member of the P-type ATPase family, and plays an essential role in regulating the cellular transmembrane ion gradient by ATP-dependent transport of Na\(^+\) and K\(^+\) across the plasma membrane.\(^1,2\) In order to maintain ion transport the Na/K-ATPase is in constant flux between two major conformation states, E1 and E2.\(^3\) The E1 state has high affinity for Na\(^+\) and ATP, while the E2 state has high affinity for K\(^+\).\(^3\) The Na/K-ATPase is composed of two noncovalently linked subunits, α and β which together form the functional unit of the enzyme.\(^2\) There are four α subunit isoforms (α1, α2, α3, and α4), and three β subunit isoform (β1, β2, and β3) all expressed in a tissue specific manner.\(^4\) The α subunit is the catalytic subunit, which contains specific binding sites for Na\(^+\), K\(^+\), ATP, and cardiotonic steroids (CTS, ligands of the Na/K-ATPase). The β subunit plays an essential role in regulating the activity of the enzyme.\(^2\) A third subunit (γ) contains the conserved FXYD motif.\(^3\) The γ subunit is not considered to be essential for enzymatic function, but has been proposed to modulate enzymatic activity.\(^5,6\)

In addition to the essential ion pumping function, elegant work from the laboratory of Dr. Xie and collaborators has shown that the cardiotonic steroid ouabain binds to the α1 subunit of the Na/K-ATPase converting it into a signal transducer capable of activating multiple protein kinase cascades.\(^7-9\) Src binds to the Na/K-ATPase α1.
subunit forming a functional signaling complex. CTS bind to the Na/K-ATPase and induce a conformational change which activates Src. Src transactivates the epidermal growth factor receptor (EGFR) which results in the activation of phospholipase C (PLC), phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinases (MAPKs), protein kinase C (PKC), and the generation of reactive oxygen species (ROS). Importantly, ouabain binding to the Na/K-ATPase induces endocytosis of the receptor complex in a manner consistent with classic receptor tyrosine kinases.

1.2 Cardiotonic Steroids and Uremic Cardiomyopathy

CTS are specific ligands for the Na/K-ATPase, and have been used to treat heart failure by coupling reduced Na/K-ATPase activity with a reduction in Na⁺/Ca²⁺-exchanger activity ultimately leading to an accumulation of intracellular sodium and increases in cytosolic calcium. This increase in cytosolic calcium results in increased cardiac contractile function. Endogenous CTS, such as ouabain and marinobufagenin (MBG), represent a relatively new class of steroid hormones. Endogenous ouabain has been postulated to be produced from the adrenal cortex and hypothalamus. In amphibians, the biosynthesis of MBG has been proposed to occur via the bile acid pathway from cholic acids. Elevated levels of endogenous CTS have been reported in a variety of clinical conditions associated with plasma volume expansion such as congestive heart failure, chronic renal failure, hypertension, renal ischemia, and preeclampsia. In animal models, administration of ouabain and MBG have been shown to cause hypertension, cardiac hypertrophy, and fibrosis. Furthermore, salt-loading in Dahl salt-sensitive rats caused an increase in brain derived ouabain, which
elevated plasma MBG levels contributing to hypertension in a process mediated by angiotension II.\textsuperscript{23}

Recent data indicates that chronic kidney disease (CKD) is prevalent, affecting up to 11\% of the US adult population.\textsuperscript{24} Platelet activation and inflammation have been implicated in the progression CKD.\textsuperscript{25} Cardiovascular disease is both common and a major cause of mortality in patients with CKD.\textsuperscript{26,27} This uremic cardiomyopathy is characterized by a decrease in diastolic function, left ventricular hypertrophy, oxidant stress, and both cardiac and renal fibrosis.\textsuperscript{4,22,28} We have shown that MBG, signaling through the Na/K-ATPase, causes many of the adverse pathological effects of experimental uremic cardiomyopathy induced by 5/6\textsuperscript{th} nephrectomy (PNx) in the rat.\textsuperscript{21} Our group has demonstrated that pharmacologic administration of MBG causes cardiac hypertrophy and fibrosis, as seen in patients, whereas active immunization against MBG attenuated this in PNx.\textsuperscript{21,22} Additionally, cardiac fibroblasts treated with MBG, at concentrations similar to those reported in experimental and clinical renal failure, has been shown to stimulate collagen production.\textsuperscript{22} This increase in collagen production appears to be dependent on the Na/K-ATPase-Src-EGFR-ROS signaling cascade.\textsuperscript{22} The transcription factor Friend leukemia integration-1 (Fli-1) has been shown to be a negative regulator of collagen synthesis.\textsuperscript{29,30} PKC-\(\delta\) phosphorylates Fli-1 and promotes collagen synthesis.\textsuperscript{31} We have shown that MBG signaling through the Na/K-ATPase, caused PKC-\(\delta\) translocation to the nucleus leading to Fli-1 phosphorylation and collagen production.\textsuperscript{32} A recent report from our lab has shown that treatment with a monoclonal antibody directed against MBG (3E9 mAb) in PNx animals resulted in a drastic decrease in blood pressure, significantly reduced cardiac levels of oxidant stress, increased the
expression of Fli-1, and caused a significant reduction in cardiac fibrosis.\textsuperscript{33}

Spironolactone and its major metabolite have been shown to competitively inhibit CTS binding to the Na/K-ATPase.\textsuperscript{34} We have shown that spironolactone treatment in both PNx animals and animals receiving MBG infusion attenuated diastolic dysfunction and cardiac fibrosis in these experimental animal models.\textsuperscript{35} Our findings indicate that spironolactone and the 3E9 monoclonal antibody may offer potential indications for the treatment of uremic cardiomyopathy.
References for Literature Review and Introduction


Chapter 2 – Manuscript

Title:
Monoclonal Antibody Against Marinobufagenin Reverses Cardiac Fibrosis in Rats With Chronic Renal Failure

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2.1 Abstract

Cardiotonic steroids (CTS) are implicated in pathophysiology of uremic cardiomyopathy. In the present study, we tested whether a monoclonal antibody (mAb) against the bufadienolide CTS, marinobufagenin (MBG), alleviates cardiac hypertrophy and fibrosis in partially nephrectomized (PNx) rats. In PNx rats, we compared the effects of 3E9 anti-MBG mAb and of Digibind, an affinity-purified digoxin antibody, on blood pressure and cardiac hypertrophy and fibrosis following 4 weeks after the surgery. In PNx rats, a fourfold elevation in plasma MBG levels was associated with hypertension, increased cardiac levels of carbonylated protein, cardiac hypertrophy, a reduction in cardiac expression of a nuclear transcription factor which is a negative regulator of collagen synthesis, Friend leukemia integration-1 (Fli-1), and an increase in the levels of collagen-1. A single intraperitoneal administration of 3E9 mAb to PNx rats reduced blood pressure by 59 mm Hg for 7 days and produced a significant reduction in cardiac weight and cardiac levels of oxidative stress, an increase in the expression of Fli-1, and a reduction in cardiac fibrosis. The effects of Digibind were similar to those of 3E9 mAb, but were less pronounced. In experimental chronic renal failure, elevated levels of MBG contribute to hypertension and induce cardiac fibrosis via suppression of Fli-1, representing a potential target for therapy.
2.2 Introduction

Uremic cardiomyopathy is a major cause of morbidity and mortality in patients with chronic kidney disease.\textsuperscript{1} Despite considerable recent progress in the understanding of the pathogenesis of uremic cardiomyopathy, there is clearly a niche for novel approaches to its treatment.\textsuperscript{1,2} An increasing body of evidence indicates that one of the factors implicated in pathogenesis of uremic cardiomyopathy is the group of hormones known as endogenous cardiotonic steroids (CTS).\textsuperscript{3} CTS regulate sodium pump activity at a cellular level and are implicated in the regulation of natriuresis and vascular tone.\textsuperscript{3} Many of the effects of these hormones appear to derive from a signaling function of the Na/K-ATPase; in particular, this signaling stimulated by CTS leads to cardiac hypertrophy and fibrosis.\textsuperscript{4,5}

Previously, we demonstrated that circulating concentrations of marinobufagenin (MBG) (14,15β-Epoxy-3β,5-dihydroxy-5β-bufa-20,22 dienolide), an endogenous bufadienolide CTS, are elevated in patients with renal failure and in partially nephrectomized rats (PNx).\textsuperscript{5,6} In PNx rats, also we observed increased cardiac and plasma levels of carbonylated proteins as well as other evidence for signaling through the Na/K-ATPase such as activation of Src and mitogen-activated protein kinase (MAPK).\textsuperscript{5,6} In these studies, active immunization of PNx rats against MBG dramatically reduced cardiac hypertrophy and fibrosis and systemic oxidant stress, as well as evidence of Na/K-ATPase signaling. Conversely, chronic administration of MBG to normotensive rats to achieve plasma concentrations of MBG as seen with PNx, produced cardiac phenotype similar to PNx.\textsuperscript{5,6}
The transcription factor, Friend leukemia integration-1 (Fli-1), a member of the ETS family, is a negative regulator of collagen synthesis,\textsuperscript{7} and reduced levels of Fli-1 were documented in skin fibroblasts of patients with scleroderma.\textsuperscript{8,9} Recent evidence indicates that suppression of Fli-1 is also implicated in profibrotic signaling by CTS. \textit{In vitro}, we have demonstrated that nanomolar concentrations of MBG stimulate collagen production by dermal, cardiac, and renal fibroblasts by a mechanism involving protein kinase C \(\delta\)-dependent phosphorylation and depletion of Fli-1.\textsuperscript{7} Interestingly, when we stably transfected renal fibroblasts with a Fli-1 expression vector which dramatically increased Fli-1 expression, the basal expression of procollagen was decreased and MBG treatment did not increase procollagen expression or appreciably reduce Fli-1 expression.\textsuperscript{7}

Recently, we developed two anti-MBG monoclonal antibodies (mAb), 3E9 and 4G4.\textsuperscript{10} In our previous experiments, 3E9 mAb exceeded 4G4 with respect to reversal of MBG-induced Na/K ATPase inhibition, and potently reduced blood pressure and restored vascular sodium pump activity in hypertensive Dahl-S rats and in pregnant Sprague-Dawley rats rendered hypertensive by NaCl supplementation. Because of these properties, in the present experiment we used 3E9 mAb for \textit{in vivo} MBG immunoneutralization, while 4G4 mAb which exhibits high affinity to MBG in competitive immunoassays was chosen for MBG measurement.\textsuperscript{10} In the present experiment, in PNx rats, we studied effects of 3E9 anti-MBG mAb on arterial pressure, cardiac fibrosis and oxidative stress, and cardiac expression of Fli-1. We also compared effects of 3E9 mAb to those of Digibind (the Fab fragments of ovine digoxin antibody) which has been demonstrated to both bind endogenous CTS,\textsuperscript{11} as well as lower blood
pressure in patients with preeclampsia,\textsuperscript{12,13} a clinical syndrome known to have elevated CTS levels.\textsuperscript{10,14}

\textbf{2.3 Methods}

\textbf{2.3.1 Animal studies}

All animal experimentation described in this article was conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals under protocols approved by the University of Toledo Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (250–300 g) were used for these studies. Eight sham nephrectomized rats comprised the control group. In 18 rats, PNx (5/6 nephrectomy) was produced by surgical removal of the right kidney and ligation of the two-thirds of the arterial supply to the left kidney as reported previously in detail.\textsuperscript{15} In brief, rats were anesthetized with a mixture of 100\% oxygen and 5\% isoflurane, an incision was made in the left flank, through which the left kidney was pulled out, and arteries supplying to upper and lower poles were ligated. After a week, the right kidney was decapsulated to avoid removal of adrenal gland, artery, vein, and ureter were ligated, and the kidney was removed. This maneuver produces sustained hypertension within 2 weeks.\textsuperscript{5,6} At 4 weeks following PNx, these rats were intraperitoneally administered vehicle ($n = 6$), Digibind ($n = 6$) or 3E9 anti-MBG mAb ($n = 6$). The dose of Digibind (10 $\mu$g/kg) was similar to that previously administered to patients with preeclampsia,\textsuperscript{12,13} and the dose of 3E9 mAb (50 $\mu$g/kg) was the same as that previously reported to reverse the EC75 to the inhibition of the Na/K-ATPase by MBG in rat renal outer medulla \textit{in vitro}, and to reduce blood pressure in hypertensive Dahl-S rats \textit{in vivo}.\textsuperscript{10} Blood pressure
was determined using the tail cuff method by IITC (Amplifier model 229, Monitor model 31, Test chamber Model 306; IITC Life Science, Woodland Hills, CA) at baseline, 3, 24, 48 h and at 1 week following antibody treatment (5 weeks after PNx). Then rats were killed and the heart weight and cardiac histology were determined. Plasma samples were stored at −80 °C for determination of CTS.

2.3.2 Oxidative stress markers

Levels of oxidative stress were assessed by measurement of protein carbonyl levels and determination of intracellular production of reactive oxygen species using the fluorescent probe dye dihydroethidium (DHE). Total protein carbonyl concentration of the plasma and left ventricular homogenate was determined by enzyme-linked immunosorbent assay using the BIOCELL PC Test kit (Northwest Life Science Specialties, Vancouver, WA). Production of reactive oxygen species was detected by DHE (Invitrogen Molecular Probes, Eugene, OR) as described previously. Briefly, left ventricle tissue was frozen in optimal cutting temperature compound, and transverse sections (10 μm) were generated with a cryostat and placed on glass slides. Tissue sections were incubated with 5 μmol/l DHE at 37 °C for 20 min according to the manufacturer’s instructions. Red fluorescence was assessed by using an Olympus FSX100 box type fluorescence imaging device (Olympus America, Center Valley, PA). The excitation wavelength was 488 nm with emission at 585 nm. Fluorescence intensity was analyzed by the use of Image J (version 1.32j) software (National Institutes of Health, USA; http://rsb.info.nih.gov/ij/).
2.3.3 Creatinine and creatinine clearance

At the conclusion of the study, 24 h urine samples were collected. At the end of urine collection, animals were killed and blood samples were obtained from abdominal aorta. Plasma creatinine was measured with a colorimetric method using a commercial kit from Teco Diagnostics (Anaheim, CA, cat. no. C515-480). Creatinine standards or plasma samples were mixed with the picric acid reagent and creatinine buffer reagent provided with the kit. The optical density value at 510 nm was measured immediately after and at 15 min. The differences between the two time points were used to calculate the creatinine concentrations. Creatinine clearance was calculated using the following formula: (urine Cr × urine Vol (ml)/plasma Cr × 24 h × 60 min).

2.3.4 Western blot analyses of Fli-1 and collagen-1

Western Blot analysis was performed on proteins from tissue homogenates as previously reported. The left ventricles from the heart were homogenized in ice-cold RIPA lysis buffer (pH 7.0) Santa Cruz Biotechnology (Santa Cruz, CA; sc-24948). The homogenate was centrifuged at 1,400g for 30 s at 4 °C. The supernatant was discarded and the pellet fraction was resuspended in 5% sodium dodecyl sulfate (SDS) and 50 mmol/l Tris-HCl (pH 7.4). The protein was quantified in the resuspended pellet fraction and was solubilized in sample buffer (2% SDS, 5% β-mercaptoethanol, 20% glycerol, 0.005% bromophenol blue, and 50mmol/l Tris- HCl; pH 7.0). The proteins, obtained from tissue homogenates, were resolved on an SDS-polyacrylamide gel electrophoresis (PAGE) using Precast Ready Gels 4–15% Tris-HCl, purchased from Bio-Rad (Hercules, CA). Ten microgram of protein per sample were loaded into each well. The proteins
from the gel were electrotransferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in 20 mmol/l Tris-HCl (pH 7.5, 150 mmol/l NaCl, and 0.1% Tween 20). Goat anti-type 1 collagen antibody (Southern Biotech, Birmingham, AL) was used to probe for collagen-1, and secondary anti-goat antibody was purchased from Santa Cruz Biotechnology. To probe for Fli-1, we used rabbit polyclonal anti-Fli1(C19) antibody (Santa Cruz Biotechnology; 1:500) and peroxidase-conjugated anti-rabbit antiserum (Amersham, Piscataway, NJ; 1:1,000). For detection, we used ECL and ECL plus purchased from Amersham Biosciences. Loading conditions were controlled using anti-actin mouse monoclonal antibody (Santa Cruz Biotechnology).

2.3.5 Histology

Trichrome staining was performed on left ventricular tissues and tissue fibrosis was quantified as previously reported.6–7,15 Left ventricle sections were immediately fixed in 4% formalin buffer solution (pH 7.2) for 18 h, dehydrated in 70% ethanol, and then embedded in paraffin and cut with a microtome. Trichrome staining was then performed and fibrosis was quantified using ImageJ software. For quantitative morphometric analysis, five random sections of trichrome slides were electronically scanned into an RGB image which was subsequently analyzed using Image J (version 1.32j) software. The amount of fibrosis was then estimated from the RGB images with a macro written by the authors (J.I.S.) by converting pixels of the image with substantially greater (>120%) blue than red intensity to have the new, gray scale amplitude = 1, leaving other pixels as with amplitude = 0.
2.3.6 MBG immunoassay

For measurement of MBG, plasma samples were extracted using C18 SepPak cartridges (Waters, Cambridge, MA). Cartridges were activated with 10 ml acetonitrile and washed with 10 ml water. Then 0.5 ml plasma samples were applied to the cartridges and consecutively eluted in the same vial with 7 ml 20% acetonitrile followed by 7 ml 80% acetonitrile and vacuum dried. Before immunoassays, samples were reconstituted in the initial volume of assay buffer. MBG was measured using a fluoroimmunoassay based on a murine anti-MBG 4G4 mAb recently described in detail. This assay is based on competition between immobilized antigen (MBG-glycoside-thyroglobulin) and MBG or other cross-reactants, within the sample for a limited number of binding sites on 4G4 anti-MBG mAbs. Secondary (goat anti-mouse) antibody labeled with nonradioactive europium was obtained from Perkin-Elmer (Waltham, MA). Data on cross-reactivity of the 4G4 mAb used for determination of MBG levels and of 3E9 mAb used for in vivo administration was reported previously. MBG (>98% high performance liquid chromatography pure) was purified from secretions from parotid glands of Bufo marinus toads as reported previously.

2.3.7 Statistical analyses

The results are presented as means ± s.e.m. Data were analyzed using one way analysis of variance followed by Newman–Keuls test (intragroup analyses), by repeated measures analysis of variance followed by Newman–Keuls test (intergroup analyses), and by two-tailed t-test (when applicable) (GraphPad Prism software, San Diego, CA). A two-sided P value of less than 0.05 was considered to be statistically significant.
2.4 Results

In Sprague-Dawley rats, PNx led to hypertension, marked increases in plasma creatinine and oxidative stress as assessed by plasma levels of carbonylated protein (Table 2.1). Plasma levels of MBG in PNx rats were elevated fourfold vs. that in sham operated animals (Figure 2-1a). Rats subjected to PNx treated with vehicle developed cardiac hypertrophy (Figure 2-2a) which was accompanied by activation of cardiac oxidative stress assessed by carbonylated protein and DHE staining (Figure 2-2b, c). Development of renal failure in rats was also associated with cardiac fibrosis assessed by computer-assisted morphological analysis and increased levels of collagen-1 in left ventricular myocardium (Figure 2-3) as we have previously reported.5,6 Cardiac levels of Fli-1 in PNx rats were markedly reduced vs. that in sham-operated animals (Figure 2-3a).

Figures 2-1 and 2-2 summarize data on the effect of administration of 3E9 anti-MBG mAb and Digibind on blood pressure, heart weight, and cardiac levels of carbonylated protein. A single administration of Digibind produced a transient decrease in arterial pressure while, in contrast, administration of 3E9 mAb resulted in a substantial and sustained decrease in systolic blood pressure following 1 week of antibody administration (Figure 2-1d). In addition to depressor effect, administration of 3E9 mAb and Digibind to PNx rats was associated with reduction in the serum levels of creatinine and a concomitant increase in creatinine clearance (Figure 2-1b, c).

Administration of both antibodies was associated with a reduction in cardiac weight and a decrease in the cardiac expression of carbonylated protein (Figure 2-2), as well as increase in the left ventricular expression of Fli-1 protein (Figure 2-3a) along with reductions in cardiac collagen 1 protein expression and morphological evidence of
fibrosis (Figure 2-3b, c). These effects of 3E9 mAb were more pronounced as compared to those of Digibind.

2.5 Discussion

The main observation of the present experiment is that a single administration of a mAb against an endogenous Na/KATPase inhibitor, MBG, to rats with experimental renal failure produced a sustained depressor effect associated with a dramatic reduction in cardiac fibrosis and increase in cardiac levels of Fli-1, a negative regulator of collagen synthesis. Fli-1 belongs to a family of Ets oncogenes, and it competes with another transcription factor, ETS-1, to maintain a balance between stimulation and repression of Colla2 gene promoter. Fli-1 is implicated in dermal fibrosis and it exhibits direct effect on collagen-1 synthesis in dermal fibroblasts, and we have observed that decreases in Fli-1 expression appear to be necessary for CTS to stimulate fibroblast collagen production.

Our present results demonstrate that blockade of the CTS-Na/ K-ATPase signal cascade can actually reverse established cardiac fibrosis in the PNx model. Our previous studies demonstrated that cardiac fibrosis was well established after 4 weeks following PNx in the Sprague-Dawley rat. In the current study, treatment was administered to rats at 4 weeks following PNx, and animals were killed 1 week later. While the vehicle treatment group, studied 5 weeks after PNx, demonstrated similar degrees of cardiac hypertrophy and fibrosis to what we had previously reported at 4 weeks, both Digibind and 3E9 treated animals had remarkable clearing of myocardial fibrosis over the next week accompanied by upregulation of cardiac Fli-1. These data further indicate that
MBG-dependent Fli-1 downregulation is implicated in the pathogenesis of cardiac fibrosis seen with experimental chronic renal failure. Our present observations that immunoneutralization of MBG was accompanied by reduction in systemic and cardiac levels of oxidative stress agrees with previous data demonstrating that generation of reactive oxygen species is implicated in MBG-dependent cell signaling.\textsuperscript{18,19}

Previous studies in PNx rats implemented remnant kidney fibrosis in the progression of renal failure in this model.\textsuperscript{20,21} Although absence of renal morphology data is a limitation of the present study, we found that in PNx rats immunoneutralization of CTS with both 3E9 mAb and Digibind reduced plasma creatinine concentration and produced a substantial increase in creatinine clearance. This observation suggests that in PNx rats beneficial effects of MBG immunoneutralization are not limited to pressor and cardiac effects, and warrants further studies of the role of CTS in the pathogenesis of renal fibrosis.

Notably, in the present study, in the case of Digibind, reduction of cardiac fibrosis occurred in the absence of a sustained blood pressure-lowering effect. Thus, in the present experiment, Digibind and 3E9 mAb in PNx rats exhibited comparable antifibrotic effects in the presence of markedly varying effects on the blood pressure within 1 week after a single injection; while the blood pressure-lowering effect of anti-MBG mAb was profound and sustained, the depressor effect of Digibind was minor and transient. We, therefore, propose that in the present study, both antibodies exhibited blood pressure independent antifibrotic effects, which agrees with our previous data demonstrating that a pronounced antifibrotic effect of active immunization of PNx rats against MBG was associated with a very minor effect on the blood pressure.\textsuperscript{5,6}
The 3E9 anti-MBG mAb employed in the current study is highly selective for bufadienolide CTS and does not cross react with cardenolide sodium pump inhibitors and other steroid hormones.\textsuperscript{10} Thus, in a competitive immunoassay, the 3E9 mAb exhibited substantial cross-reactivity only with two bufadienolides, telocinobufagin, a possible precursor of MBG which was reported to be elevated in plasma of patients with uremia,\textsuperscript{22} and cinobufotalin which differs from MBG in having one extra hydroxyl group.\textsuperscript{10} Previously, we reported that following high-performance liquid chromatography-fractionation of CTS from preeclamptic placentae, a competitive immunoassay based on Digibind exhibited reactivity to high-performance liquid chromatography fractions having retention times similar to that seen with MBG and other bufadienolides, but not to ouabain-like immunoreactive material.\textsuperscript{23} Most recently, in patients with chronic kidney disease and in PNx rats we demonstrated that increase in plasma CTS detected by Digibind is likely to reflect an increase in the levels of MBG.\textsuperscript{24} These observations suggest that in renal failure MBG represents a target for Digibind.

In conclusion, in experimental chronic renal failure, elevated levels of MBG contribute to hypertension and induce cardiac fibrosis via suppression of Fli-1, representing a potential target for therapy. The effectiveness of 3E9 mAb for reversing the cardiac disease in PNx animals and the fact that 3E9 mAb exhibits a long-lasting effect following a single injection, suggests a potential role for MBG immunoneutralization in patients with uremic cardiomyopathy.
2.6 Manuscript References


2.7 Table and Figure Legends

**Table 2.1.** Physiological measurements in control and PNx rats. Means ± SEM. *- P<0.01 vs. control group. Two-tailed t-test or Wilcoxon test (plasma carbonylated protein). PNx, partially nephrectomized rats. *P<0.01 vs. control group.

**Figure 2-1.** Plasma levels of MBG (A), creatinine (B), and creatinine clearance (C) in sham-operated (Sham) and PNx rats, treated with vehicle (Veh), Digibind (DG) or anti-MBG mAb (3E9). Effects of administration of 3E9 anti-MBG mAb and of Digibind to PNx rats on systolic BP (D). Means ± SEM from 6 observations. A: (*) - P<0.01 vs. Sham by two-tailed t-test. B and C: (*) - P<0.05, (**) - P<0.001 vs. Sham, (#) - P<0.05, (##) - P<0.01 vs. vehicle by one-way ANOVA followed by Newman-Keuls test. D: By repeated measures ANOVA and Newman-Keuls test: Digibind vs. vehicle – P<0.05; 3E9 mAb vs. vehicle – P<0.01; Digibind vs. 3E9 – P<0.01.

**Figure 2-2.** Effects of administration of 3E9 anti-MBG mAb and of Digibind to PNx rats on heart weight (A), and on cardiac levels of oxidative stress assessed by measurement of carbonylated protein (B) and DHE fluorescence intensity (C); upper panels, representative measurements; lower panel – quantitative measurements, mean±SEM of 4 densiometry determinations). PNx – partially nephrectomized rats. Vehicle – PNx rats administered vehicle. Means ± SEM from 6 observations. A-C: By one-way ANOVA and Newman-Keuls test (*) –
P<0.05 and (**) – P<0.01 vs. Sham. (#) – P<0.05 and (##) – P<0.01 vs. vehicle treated PNx rats.

Figure 2-3. Representative (upper panel) and quantitative (lower panel, mean±SEM of 4 densiometry measurements) analysis of Fli-1 (A) and collagen-1 (B) Western blots performed on cardiac tissues from the different groups. Actin was used to control loading. C – representative (upper panel) and quantitative (lower panel, mean±SEM of 4 densiometry measurements) trichrome-stained photomicrographs obtained from cardiac tissue derived from the different experimental groups. Sham – sham-operated rats, PNx – partially nephrectomized rats, Veh – PNx rats administered vehicle, DG – PNx rats administered Digibind, 3E9 – PNx rats administered 3E9 anti-MBG mAb. By one-way ANOVA and Newman-Keuls test: (*) – P<0.01 vs. Sham; (#) – P<0.05, (##) – P<0.01 vs. Veh.
2.8 Table and Figures

Table 2.1

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Figure 2-1.
Figure 2-2.
Figure 2-3.
Chapter 3 – Manuscript

Title:

Rapamycin Reduces Cardiac Fibrosis in Experimental Uremic Cardiomyopathy

Authors:

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To be Submitted to Hypertension
3.1 Abstract

**Background:** We have shown that experimental uremic cardiomyopathy causes cardiac fibrosis and is associated with increased levels of the cardiotonic steroid marinobufagenin (MBG), an inhibitor of the Na/K-ATPase. The mammalian target of rapamycin (mTOR) is a serine/threonine kinase implicated in the progression of many different forms of renal disease. Treatment with rapamycin (an mTOR inhibitor) has been shown to attenuate inflammation, and renal fibrosis in experimental models of renal disease. The use of rapamycin in the setting of experimental uremic cardiomyopathy has not been defined.

**Materials and Methods:** Male Sprague Dawley rats weighing between 250-300 gms were used for these studies. Rats were divided into six groups. In the first group, partial nephrectomy (PNx) was performed as we have previously described. This maneuver produces sustained hypertension by 2 weeks under these conditions. In the second group, PNx was performed and rapamycin was administered (0.2mg/kg/day). The third group received both rapamycin (0.2mg/kg/day) and MBG (10µg/kg/day). The fourth and fifth groups were administered MBG alone and rapamycin alone. The sixth group consisted of sham operated controls. All treatments were performed for 4 weeks with the use of osmotic minipumps.

**Results:** The PNx animals showed an extensive increase in plasma MBG levels, systolic BP, and cardiac fibrosis. Plasma MBG levels were significantly decreased in the PNx-rapamycin animals compared to PNx (124 ± 15 vs 342 ± 20, P<0.01). The PNx-rapamycin animals showed a substantial decrease in cardiac fibrosis compared to PNx
animals. MBG treated animals had significant increases in systolic BP, and cardiac fibrosis compared to controls. Rapamycin treatment in combination with MBG did not significantly attenuate these effects.

**Conclusion:** The mTOR pathway has been implicated in the generation of renal fibrosis during renal failure. Our results suggest that the mTOR pathway may have a significant impact in the generation of cardiac fibrosis. Treatment with rapamycin may provide a novel therapy for reducing cardiac fibrosis in the setting of uremic cardiomyopathy.
3.2 Introduction

The high mortality rate in patients with chronic renal failure is ultimately due to severe cardiovascular disease. This uremic cardiomyopathy is characterized by cardiac hypertrophy, diastolic dysfunction, and cardiac fibrosis along with elevated circulating concentrations of the cardiotonic steroid marinobufagenin (MBG), a ligand of the Na/K-ATPase. MBG belongs to a family of bufadienolides previously described in amphibians. In toads, the biosynthesis of MBG occurs via the bile acid pathway from cholic acids. This pathway may also be responsible for the production of MBG in mammals. We have shown that MBG is elevated in patients with renal failure and in rats subjected to partial nephrectomy (PNx), and those with pharmacologic administration of MBG developed a similar cardiomyopathy as seen in patients, whereas active immunization against MBG attenuated this in PNx. In PNx rats, we also observed increased cardiac and plasma levels of carbonylated proteins as well as evidence for signaling through the Na/K-ATPase such as activation of Src and mitogen-activated protein kinase (MAPK). A recent report from our group has shown that treatment with a monoclonal antibody directed against MBG drastically reduced cardiac fibrosis in PNx animals.

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase composed of two signaling complexes, mTORC1 and mTORC2. The mTORC1 complex is involved in cellular proliferation and growth, while mTORC2 is involved the regulation of the cytoskeleton. The mTOR pathway has been implicated in the progression of many different forms of renal disease including experimentally induced uremic cardiomyopathy. Treatment with rapamycin (an mTORC1 inhibitor) has been
shown to attenuate inflammation, fibrosis, and cardiac hypertrophy in experimental models of renal disease. Rapamycin is also a competitive inhibitor of CYP27A1, a key rate limiting enzyme of the bile acid pathway.

Based on this background, the primary goals of the present study were to determine the effects of rapamycin on cardiac fibrosis and MBG production using the rat PNx model of uremic cardiomyopathy.

3.3 Methods

3.3.1 Animal Studies

All animal experimentation described in this article was conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals under protocols approved by the University of Toledo Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (250–300 g) were used for these studies. Rats were divided into six groups. In the first group, partial nephrectomy (PNx) was performed as we have previously described. In the second group, PNx was performed and rapamycin (RAPA) was administered (0.2mg/kg/day). The third group received both rapamycin (0.2mg/kg/day) and MBG (10µg/kg/day). The fourth and fifth groups were administered MBG alone (10µg/kg/day) and rapamycin alone (0.2mg/kg/day). The dose of MBG is the same as we have previously reported to induce the physiological changes associate with uremic cardiomyopathy. The sixth group consisted of sham
operated controls. All treatments were performed for 4 weeks with the use of osmotic minipumps (Alzet, model 2004). Minipumps were inserted SC through a flank incision.

3.3.2 Blood Pressure, Cardiac Physiology, and Other In Vivo Measurements

Blood pressure was determined using the tail cuff method by IITC (Amplifier model 229, Monitor model 31, Test chamber Model 306; IITC Life Science, Woodland Hills, CA) at baseline, and once weekly for four weeks. Then rats were euthanized and the heart weight and cardiac histology were determined. Plasma samples were stored at −80 °C for biochemical analysis. Plasma MBG and creatinine were measured as we have previously described. 6, 13

3.3.3 Oxidative stress markers

Levels of oxidative stress were assessed by determination of intracellular production of reactive oxygen species using the fluorescent probe dye dihydroethidium (DHE). Production of reactive oxygen species was detected by DHE (Invitrogen Molecular Probes, Eugene, OR) as described previously. 14, 15 Briefly, left ventricle tissue was frozen in optimal cutting temperature compound, and transverse sections (10 μm) were generated with a cryostat and placed on glass slides. Tissue sections were incubated with 5 μmol/l DHE at 37 °C for 20 min according to the manufacturer’s instructions. Red fluorescence was assessed by using an Olympus FSX100 box type fluorescence imaging device (Olympus America, Center Valley, PA). The excitation
wavelength was 488 nm with emission at 585 nm. Fluorescence intensity was analyzed by the use of Image J (version 1.32j) software (National Institutes of Health, USA; http://rsb.info.nih.gov/ij/).

3.3.4 Isolation of Cardiac Fibroblasts and JEG-3 Cell Experiments

Isolation of cardiac fibroblasts was carried out as previously described by Brilla and coworkers 7 with modifications as previously reported. 4 Briefly, male Sprague Dawley rats weighing 250-300 grams were used to obtain fibroblast from the hearts. The rats were anesthetized with pentobarbital (50 mg/kg), and their hearts were removed and perfused under sterile condition via the ascending aorta with Joklik's medium (Sigma-Aldrich, St. Louis, MO) in a modified Langendorff apparatus. After 5 min of perfusion, the perfusate was placed in Joklik's medium containing 0.1% collagenase type 2 (Worthington Biochemical, Lakewood, NJ) and 0.1% BSA which was circulated for 15-25 min until the heart became flaccid. Ventricles were excised and finely cut, and shaken in Joklik's modified medium with 0.1% collagenase and 0.1% BSA for 15 min. Cells/tissue suspension was allowed to settle for 15 min and was centrifugated at 500 rpm for 10 min. The supernatant then was centrifugated at 1500 rpm for 15 min. The resulting pellet was suspended in DMEM supplemented with antibiotics (penicillin/streptomycin/fungizone) plus 15% FBS (Hyclone, Logan, UT) and seeded onto plates and incubated for 1 hr. Unattached cells were removed, and the attached fibroblasts cells were allowed to grow until confluence and then trypsinized and passaged once at 1:3 dilution. Cells were allowed to grow confluent prior to use for experimental
purposes. All cells used in these experiments were from passage one unless otherwise specified. Human placental chorionic epithelial cells (JEG-3) were purchased from a commercially available vendor (ATCC), cultured in 6-well plates, and grown to confluence in minimum essential media over 48 hours. Cells were cultured in 2.5% FBS media and rapamycin (1μM) was added into half the wells. Both control and rapamycin-treated cells were sampled after 3, 6, and 12 hours of incubation. MBG was extracted from the collected media using C18 as reported. Competitive immunoassays were performed using a monoclonal anti-MBG antibody to determine the concentration of MBG in the samples.

3.3.5 Western Blot Analysis

Western blot analysis was performed on proteins from tissue homogenates as previously reported. For the cell lysates, the cells were grown to confluence and starved for 18 h in DMEM with 1% FBS. The cells then were treated with MBG or rapamycin for 24 h when looking for procollagen expression. The cells were washed with phosphate buffered saline (BPS) twice and exposed to lysis buffer. For detection of Collagen-1, the left ventricles from the heart were homogenized in ice-cold RIPA lysis buffer (pH 7.0) Santa Cruz Biotechnology (Santa Cruz, CA; sc-24948). The homogenate was centrifuged at 1,400g for 30 s at 4 °C. The supernatant was discarded and the pellet fraction was resuspended in 5% sodium dodecyl sulfate (SDS) and 50 mmol/l Tris-HCl (pH 7.4). The protein was quantified in the resuspended pellet fraction and was solubilized in sample buffer (2% SDS, 5% β-mercaptoethanol, 20% glycerol, 0.005%
bromophenol blue, and 50mmol/l Tris- HCl; pH 7.0). The proteins, obtained from tissue homogenates, were resolved on an SDS-polyacrylamide gel electrophoresis (PAGE) using Precast Ready Gels 4–15% Tris-HCl, purchased from Bio-Rad (Hercules, CA). Ten microgram of protein per sample were loaded into each well. The proteins from the gel were electrotransferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in 20 mmol/l Tris-HCl (pH 7.5, 150 mmol/l NaCl, and 0.1% Tween 20). Goat anti-type 1 collagen antibody (Southern Biotech, Birmingham, AL) was used to probe for collagen-1, and secondary anti-goat antibody was purchased from Santa Cruz Biotechnology. For detection, we used ECL and ECL plus purchased from Amersham Biosciences. Loading conditions were controlled using anti-actin mouse monoclonal antibody (Santa Cruz Biotechnology).

3.3.6 Histology

Left ventricle sections were immediately fixed in 4% formalin buffer solution (pH 7.2) for 18 h, dehydrated in 70% ethanol, and then embedded in paraffin and cut with a microtome. Fast green staining with sirius red (0.1%) was performed on left ventricular tissues as described previously, and fibrosis quantified using ImageJ software (ImageJ 1.36b, National Institutes of Health, USA). For confirmation of the histological findings, quantitative determination of Collagen-1 in left ventricular homogenates was performed using Western blot (as described above).
3.3.7 Statistical Analysis

The results are presented as means ± s.e.m. Data were analyzed using one way analysis of variance followed by Newman–Keuls test (intragroup analyses), by repeated measures analysis of variance followed by Newman–Keuls test (intergroup analyses), and by two-tailed t-test (when applicable) (GraphPad Prism software, San Diego, CA). A two-sided P value of less than 0.05 was considered to be statistically significant.

3.4 Results

3.4.1 Effect of Rapamycin on Blood Pressure, MBG Levels

Rapamycin treatment alone demonstrated a slight elevation in systolic BP, but did not significantly alter MBG levels compared to control animals. PNx surgery substantially increased the heart weight/body weight ratios. PNx surgery and MBG infusion produced sustained hypertension throughout the duration of the experiment. PNx MBG levels were similar to the MBG levels produced by MBG infusion alone. PNx surgery with rapamycin infusion showed a significant decrease in systolic BP by the third week which persisted at four weeks compared to PNx alone. The PNx surgery with rapamycin treatment also demonstrated a drastic decrease in MBG levels. Coadministration of MBG with rapamycin did not significantly attenuate systolic BP or plasma MBG levels. These data are summarized in Table 3.1.
3.4.2 Effect of Rapamycin on Cardiac Fibrosis and oxidative stress

Cardiac fibrosis was assessed in the left ventricular myocardium by histological analysis (sirius red with fast green staining) and collagen 1 expression determined by Western blot. Both PNx and MBG infusion resulted in substantial increases of collagen expression and cardiac scarring, while PNx with rapamycin infusion drastically lowered these effects (Figure 3-1 A and B). Coadministration of rapamycin with MBG did not alter MBG induced cardiac fibrosis (Figure 3-1 A, and B). The increase in cardiac fibrosis in PNx and MBG treated animals was accompanied with an increase in cardiac oxidative stress as measured by DHE staining (Figure 3-2). Treatment with rapamycin did not alter oxidative stress in these animals.

3.4.3 Effect of Rapamycin on Fibroblast Procollagen Expression

Cultured cardiac fibroblasts treated with 1 and 100 nM of MBG resulted in a significant increase in procollagen 1 expression determined by Western blot (Figure 3.3). Treatment with rapamycin (10.9 and 109 pM) significantly attenuated MBG (1 and 100 nM) induced procollagen 1 expression (Figure 3-3.) The concentrations of rapamycin chosen have been reported to have little effect on cell viability.¹⁷
3.4.4 Effect of Rapamycin on MBG Production by JEG-3 Cells

Cultured human placental chorionic epithelial cells (JEG-3), which produce MBG, were treated with 1 µM of rapamycin to test the effects on MBG production. Rapamycin treatment (1 uM) at 3 and 6 hours significantly reduced MBG production compared to controls (84 vs 60 pmol/g protein, p<0.01; and 243 vs 116, pmol/g protein, p<0.01, Figure 3-4).

3.5 Discussion

The mTOR pathway has been shown to play a pivotal role in several different forms of renal disease. Treatment with rapamycin attenuates many of the physiological changes associated with a decrease in renal function, including interstitial fibrosis. Our current work demonstrates that rapamycin treatment in the setting of experimental uremic cardiomyopathy significantly reduces cardiac fibrosis. This is in support of a recent report with similar findings in a murine model of uremic cardiomyopathy. We have also shown that fibroblast treatment with rapamycin drastically reduced procollagen production in the presence of MBG. Importantly, rapamycin also drastically lowered MBG levels in both the PNx model and in human placental chorionic epithelial cells (JEG-3).

Recent work in animal models of renal disease has provided compelling evidence for the involvement of mTORC1 in the generation of fibrosis. In an animal model of unilateral obstructive nephropathy, as well as in fibroblasts, the profibrotic cytokine
TGF-β was shown to activate mTORC1 acting through a PI3K pathway.\textsuperscript{18} In human fibroblasts, the mTOR pathway has been shown to regulate collagen type I production.\textsuperscript{19} Treatment with rapamycin has been shown to decrease TGF-β, fibroblast proliferation, and renal fibrosis.\textsuperscript{18,20,21} In support of our results, rapamycin treatment significantly decreased cardiac fibrosis as evaluated by trichrome staining in a murine model of uremic cardiomyopathy.\textsuperscript{10} Similar results were reported using a transverse aortic constriction model.\textsuperscript{22}

We have shown that MBG causes many of the pathophysiological changes associated with experimental uremic cardiomyopathy including cardiac fibrosis\textsuperscript{5}, and that MBG induces cardiac fibroblasts to produce collagen.\textsuperscript{4} The transcription factor Friend leukemia integration-1 (Fli-1) acts as a negative regulator of collagen production\textsuperscript{23,24}, and activation of protein kinase C-delta (PKC-\delta) can phosphorylate Fli-1 to promote collagen synthesis.\textsuperscript{25} We have recently reported that MBG induces translocation of PKC-delta, which phosphorylates Fli-1 and leads to an increase in collagen synthesis.\textsuperscript{26} Signaling through PKC-delta has been shown to activate the mTOR pathway.\textsuperscript{27}

Treatment with rapamycin significantly reduced circulating MBG levels compared to PNx animals. In addition, treatment with rapamycin in JEG-3 cells, which produce MBG, resulted in a 52% reduction in MBG levels after six hours of treatment. Endogenous cardiotonic steroids have been postulated to be synthesized from the classic steroidogenesis pathway through cholesterol side-chain cleavage and pregnenolone precursors.\textsuperscript{2} Though this theory is still upheld for other cardiotonic steroids such as
ouabain, there have been controversial results with regard to MBG production.\textsuperscript{2} In toads, the biosynthesis of MBG occurs via the bile acid pathway form cholanic acids.\textsuperscript{2} Rapamycin acts as a competitive inhibitor of CYP27A1, a key rate-limiting enzyme of the bile acid pathway. Our data provides preliminary evidence indicating that the drastic reduction in MBG levels in both PNx animals and JEG-3 cells may be due to competitive inhibition of CYP27A1. Thus, in the setting of experimental uremic cardiomyopathy, rapamycin may have a duel effect of both inhibiting cardiac fibrosis and reducing MBG production.

Importantly, we did not see a significant reduction in cardiac fibrosis or MBG levels with combined MBG infusion and rapamycin treatment. We also did not see a reduction in oxidant stress in PNx animals treated with rapamycin or combined MBG and rapamycin treatment. We speculate that rapamycin was unable to provide a therapeutic effect in the setting of continuous MBG infusion. Furthermore, MBG induced ROS production may precede mTOR activation. Future experiments are warranted in order to determine if MBG is produced by the bile acid pathway in mammals, and if higher doses of rapamycin would overcome the effects of continuous MBG infusion.

In conclusion, the mTOR pathway has been implicated in the generation of renal fibrosis during renal failure. Our results suggest that the mTOR pathway may have a significant impact in the generation of cardiac fibrosis. Treatment with rapamycin may provide a novel therapy for reducing MBG levels and cardiac fibrosis in the setting of uremic cardiomyopathy.
3.6 Manuscript References


3.7 Table and Figure Legends

**Table 3.1.** Effects of rapamycin on physiological measurements after PNx or infusion of MBG. Sham refers to animals subject to sham surgery; PNx refers to partial nephrectomy; PNx + Rapa refers to PNx surgery and rapamycin infusion using minipumps; Rapa refers to rapamycin infusion using minipumps; MBG + Rapa refers to coadministration of MBG and rapamycin using minipumps; and MBG refers to MBG infusion using minipumps.*p<0.05 vs sham, †p<0.01 vs sham, §p<0.01 vs PNx, ‡p<0.01 vs MBG, ¶p<0.01 vs PNx + Rapa, ‖p<0.01 vs MBG + Rapa

**Figure 3-1.** A, Representative (top) and quantitative analysis of collagen 1 (mean±SEM) Western blots performed on cardiac tissue from the different groups. Actin was used as a loading control. B, Representative Sirius red and Fast green stained photomicrographs obtained from cardiac tissue derived from the different experimental groups. Amount of fibrosis expressed as mean±SEM measured using computer-assisted morphometry, as we have previously described 5. Sham refers to animals subject to sham surgery (n=8); PNx refers to partial nephrectomy (n=10); PNx + Rapa refers to PNx surgery and rapamycin infusion using minipumps (n=6); Rapa refers to rapamycin infusion using minipumps (n=8); MBG + Rapa refers to coadministration of MBG and rapamycin using minipumps (n=8); and MBG refers to MBG infusion using minipumps (n=8). *p<0.05 vs Sham, **p<0.01 vs Sham, #p<0.01 vs PNx, ##p<0.01 vs Rapa, †p<0.05 vs Rapa.
Figure 3-2. Effects of rapamycin treatment on cardiac levels of oxidative stress assessed by DHE fluorescence intensity: (upper panels, representative measurements; lower panel – quantitative measurements, mean±SEM of 4 densitometry determinations). Sham refers to animals subject to sham surgery; PNx refers to partial nephrectomy; PNx + Rapa refers to PNx surgery and rapamycin infusion using minipumps; Rapa refers to rapamycin infusion using minipumps; MBG + Rapa refers to coadministration of MBG and rapamycin using minipumps; and MBG refers to MBG infusion using minipumps. *p<0.01 vs Sham, **p<0.01 vs Rapa.

Figure 3-3. Representative Western blot against procollagen 1 derived from cardiac fibroblasts treated with MBG (1 or 100 nM), rapamycin (10.9 or 109 pM), or a combination with the corresponding quantitative data shown as the mean±SEM of 5 experiments. *P<0.01 vs control, **p<0.01 vs MBG 100 nM, #P<0.01 vs MBG 1 nM

Figure 3-4. MBG production in JEG-3 cells after incubation with 1uM rapamycin for 3 and 6 hours. *p<0.05 vs control, **p<0.01 vs control.
### 3.8 Table and Figures

**Table 3.1**

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Figure 3-1.

A

Collagen-1

Actin

B

Sham | Rapa | PNx | PNx + Rapa | MBG | MBG + Rapa

Collagen-1 Expression (Fraction of Control)

Fibrosis (% Area)
Figure 3-2.
Figure 3-3.

Procollagen-1

Actin

Procollagen-1 Expression (Relative Units)
Figure 3-4.
Chapter 4 – Manuscript

Title:
Platelet Activation in Patients with Atherosclerotic Renal Artery Stenosis Undergoing Stent Revascularization

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4.1 Abstract

**Background and Objectives:** Soluble CD40 ligand (sCD40L) is a marker of platelet activation; whether platelet activation occurs in the setting of renal artery stenosis and stenting is unknown. Additionally, the effect of embolic protection devices and glycoprotein IIb/IIIa inhibitors on platelet activation during renal artery intervention is unknown.

**Design, setting, participants, & measurements:** Plasma levels of sCD40L were measured in healthy controls, patients with atherosclerosis without renal stenosis, and patients with renal artery stenosis before, immediately after, and 24hr after renal artery stenting.

**Results:** Soluble CD40L levels were higher in renal artery stenosis patients than normal controls (347.5 ± 27.0 vs 65.2 ± 1.4 pg/ml, p<0.001), but were similar to patients with atherosclerosis without renal artery stenosis. Platelet rich emboli were captured in 26% (9/35) of embolic protection device patients and in these patients sCD40L was elevated prior to the procedure. Embolic protection device use was associated with a non-significant increase in sCD40L whereas sCD40L declined with abciximab post-procedure (324.9 ± 42.5 vs 188.7 ± 31.0 pg/ml, p=0.003) and at 24hrs.

**Conclusions:** Atherosclerotic renal artery stenosis is associated with platelet activation but this appears to be related to atherosclerosis, not renal artery stenosis specifically. Embolization of platelet rich thrombi is common in renal artery stenting and is inhibited with abciximab.
4.2 Introduction

Platelet activation leading to thrombus formation is a well-described complication of coronary artery disease, yet its occurrence in renal artery stenosis (RAS) is unknown. 1-4 RAS is a major cause of secondary hypertension and an important cause of renal failure.1-3, 5, 6 Although the utility of stent revascularization in patients with RAS is uncertain, several studies suggest that at least a portion of patients develop a loss of kidney function post-procedure. 1-3 6, 7

Soluble CD40 ligand (sCD40L) is expressed and secreted by platelets after activation and plays a vital role in the immune, inflammatory, and coagulative responses following injury or stress and in the setting of transplantation has been linked to renal fibrosis.8-15 Moreover, high levels of sCD40L correlate with cardiovascular events in patients with unstable coronary syndromes.13, 16-18 GP IIb/IIla inhibitors may lower the level of platelet activation in vitro and the level of sCD40L released from platelets upon activation.19, 20 A recent report from our group has demonstrated that the use of a GPIIb/IIla inhibitor in combination with an embolic protection device (EPD) during renal artery stenting may improve renal function following the revascularization procedure.21 However the relationship between platelet activation and patient outcome following renal artery stenting is uncertain.

On this background, the goals of the present study were to determine 1) if platelet activation is associated with atherosclerotic RAS, and 2) whether platelet activation occurs during renal artery stenting, and 3) if platelet thrombus formation captured by the EPD correlates with systemic platelet activation.
4.3 Materials and Methods

The study, ClinicalTrials.gov identifier NCT00234585, was conducted with funding provided by the sponsors, but study conduct, analysis, and reporting performed independent of the sponsors. ICH good clinical practice guidelines were followed with patients providing informed consent in an IRB approved protocol.

Platelet activation levels from the RAS patients were compared to 30 healthy controls and, 30 patients with atherosclerosis undergoing coronary angiography, but free of RAS. A total of 100 RAS patients were recruited from 7 sites. Inclusion required a history of hypertension, renal insufficiency, heart failure, or angina with poorly controlled hypertension and also the presence of 1 or more stenoses, ≥ 50% and <100, treatable with the EPD. RAS patients were randomized to the following allocations: 1/2 to Angioguard, 1/2 to no Angioguard; 1/2 to abciximab, 1/2 to placebo infusion yielding four groups: control, Angioguard only, abciximab only, and Angioguard with abciximab.

4.3.1 Pre-procedural care

In patients with RAS prior to double-blinded administration of Abciximab or placebo, systolic blood pressure was lowered to ≤160 mmHg. The target ACT was 275 seconds, if randomized to the EPD device an ACT of >300 seconds was required. A bolus of 0.25 mg/kg abciximab (or placebo) was administered 5 minutes before crossing the lesion, and was followed by an infusion at 0.125 µg/kg/min (maximum 10 µg /min) for 12 hours.
4.3.2 Central Laboratory Analysis

The blinded analysis of EPD contents was performed by the CV Path core lab (Gaithersburg, MD). Platelet emboli consisted of layered platelet aggregates with varying amounts of entrapped leukocytes and fibrin as evidence on hematoxylin and eosin stained sections.\(^{22}\) Glomerular filtration rate (GFR), calculated from the modified MDRD equation\(^{23}\), was used as the primary measure of renal function. Creatinine was measured by a modified Jaffé reaction using the IDMS-traceable assay at the University of Minnesota Core Lab for all subjects.

4.3.3 Blood Collection

Peripheral venous blood was collected at baseline, immediate post, and 24hr post procedure in lithium heparin plasma separator tubes, spun at 1000 x g for 15 minutes, and frozen at –80°C until batch analysis.

4.3.4 Measurement of soluble CD40 ligand

Plasma levels of soluble CD40 ligand (sCD40L) were measured by enzyme linked immunosorbent assay (ELISA, R&D Systems; Minneapolis, MN). The ELISA kit had intra-assay and interassay coefficients of 5% and 6%, respectively. The average minimum detectible amount of sCD40L was 4.2 pg/ml.
4.3.5 Statistical Analysis

Study data are presented as continuous (mean±SEM) and categorical data. Statistical analysis was performed on subjects with complete data for platelet activation measurements at the baseline, immediate post, and 24hr post procedure time points (n=84). SAS one-way ANOVA were used to test for significance among groups. Paired t-tests and Fisher’s PLSD post hoc tests were used to test for significance between groups. Unpaired t-tests were used to test for significance between the normal subjects, patient controls, and the RAS patients. Significance was defined as P<0.05. All analyses were performed in SAS or JMP.

4.4 Results

Baseline characteristics of the normal controls (n=30), atherosclerotic controls (n=30), and the RAS patients (n=84), are shown in Table 4.1. The RAS patients had a significantly higher level of sCD40L compared to normal controls (347.5 ± 27.1 vs 65.2 ± 1.4 pg/ml, P<0.001) (Figure 4-1). However, sCD40L levels were similar when compared to the patients with atherosclerosis who were free of renal artery stenosis (347.5 ± 27.1 vs 335.2 ± 38.6 pg/ml, P=0.79) (Figure 4-1). Soluble CD40L, either at baseline or after the stenting, was not associated with baseline GFR or subsequent changes in kidney function.
4.4.1 EPD Content, Platelet Embolization, and sCD40L

Twenty six percent (9/35) of the patients that received the Angioguard had platelet rich emboli captured within the filter. In these patients with platelet rich emboli, sCD40L levels were higher than in patients without platelet emboli both before (497.9 ± 105.0 vs 313.7 ± 28.4 pg/ml, p=0.02) and after the procedure (443.3 ± 111.3 vs 232.2 ± 32.4 pg/ml, p=0.02) (Figure 4-2).

4.4.2 Effect of Distal Protection and Drug Treatment

Patients with RAS randomized to abciximab had a significant decrease in sCD40L levels immediately following the procedure (324.9 ± 42.5 vs 188.7 ± 31.0 pg/ml, p=0.003), which persisted at 24hrs (324.9 ± 42.5 vs 181.2 ± 19.3 pg/ml, p=0.002) (Figure 4.3). In patients randomized to the Angioguard, sCD40L levels rose slightly immediately following the procedure and at 24hrs (p=0.90) (Figure 4-3). Patients randomized to both the Angioguard device and abciximab showed a significant decrease in sCD40L immediately following the procedure (322.8 ± 35.2 vs 203.6 ± 33.1 pg/ml, p=0.03) but this difference was no longer significant at 24 hours (Figure 4-3).

4.4.3 Effect of Abciximab and Clopidogrel

Clopiogrel use was not associated with lower sCD40L at baseline. For patients on clopidogrel before intervention, sCD40L levels rose slightly immediately following the procedure and decreased at 24hrs (p=0.53). For patients randomized to Abciximab and not taking clopidogrel, sCD40L levels decreased significantly immediately following the procedure (310.5 ± 33.0 vs 195.2 ± 31.3 pg/ml, p=0.008), which persisted at 24hrs (310.5...
± 33.0 vs 173.1 ± 18.7 pg/ml, p<0.001. Patients on clopidogrel and randomized to Abciximab showed a significant decrease in sCD40L at immediate post procedure (346.3 ± 48.1 vs 198.3 ± 30.6, p=0.02). Similar effects were seen in patients that were prescribed clopidogrel on the day of procedure.

4.5 Discussion

Platelet activation is a major cause of events and complications in coronary artery disease and with coronary revascularization.24, 25 The use of platelet inhibitors during coronary stenting reduces the potentially harmful effects of platelet activation including abrupt vessel occlusion, myocardial infarction, and stent thrombosis.25 To date, the extent of platelet activation, and effect of anti-platelet therapies in the setting of renal artery stenting has not been established. Thus, in the current study we sought to determine whether atherosclerotic renal artery stenosis was associated with platelet activation and the effect(s) of embolic protection and or use of platelet inhibitors on markers of platelet activation.

Increased platelet activation is associated with a variety of vascular disorders including acute coronary syndromes, stable coronary artery disease, and restenosis following percutaneous coronary intervention.26, 27 Soluble CD40L is a particularly attractive marker for platelet activation since it is shed from the surface of activated platelets, is easily measured, and meaningfully participates in a number of important biologic processes including activation of immunity and thrombosis.28 The current study found increased levels of sCD40L in the setting of RAS, however, this appears to be a non-specific association with atherosclerosis in general as opposed to being attributable
to RAS specifically. More importantly though increased levels of sCD40L prior to the procedure were more likely to have embolization of platelet-rich thrombi and these patients had persistently elevated levels of sCD40L after the procedure. This finding may represent a potentially modifiable feature denoting increased risk for patients referred for renal artery revascularization.

The current study also demonstrated that abciximab effectively inhibits platelet activation, as denoted by substantial suppression of sCD40L, up to 24 hrs following the procedure. Others have also observed the ability of GPIIb/IIIa inhibitors to lower levels of sCD40L in settings such as acute coronary syndromes and in STEMI patients undergoing coronary intervention. 29, 30 The current finding extends the prior observation that a GPIIb/IIIa inhibitor, when combined with an embolic protection device to capture atheroembolic debri, resulted in the most favorable renal function outcome. 21

The suppression of sCD40L release, observed with abciximab administration in the current study, creates a plausible biologic pathway to explain the observation that abciximab use was associated with improved renal function after stenting. In the kidney the sCD40L/CD40 may be directly responsible for renal injury. Previously, others have shown that angiotensin II stimulates release of renal TGF-β that in turn increases expression of the CD40 receptor on the proximal tubule of the kidney. 31 Pontrelli et al. has shown that CD40 cross-linking on proximal tubular epithelial cells is pro-inflammatory and induces fibrosis by stimulating the expression of plasminogen activator inhibitor-1 (PAI-1) acting through a signaling pathway which is independent of the proinflammatory signaling effects of CD40L. 15 In addition, activation of the CD40
receptor results in infiltration of inflammatory cells into the interstitium of the kidney through monocyte chemoattractant protein-1 (MCP-1) and intercellular adhesion molecule-1 (ICAM-1) expression. IL-8 amplifies CD40/CD154-mediated ICAM-1 production via the CXCR-1 receptor and p38-MAPK pathway in human renal proximal tubule cells. Furthermore, inhibition of the CD40/CD40L significantly decreased the severity of renal injury in an animal model of chronic proteinuric renal disease. Thus, it is conceivable that in patients with renal ischemia 1) the CD40 receptor is over-expressed due to angiotensin II stimulation, 2) sCD40L shed by locally activated platelets may activate the receptor and stimulate peritubular fibrosis in a manner independent of renal blood flow or ischemia, and 3) this process may be accelerated at the time of a stent procedure. In this regard the association between the GP IIb/IIIa inhibitor Abciximab and improved renal function outcomes observed in the RESIST study may be attributable to the drug’s effects in suppressing sCD40L as opposed to an effect on thrombosis per se.

An observation from the RESIST study was that the EPD, when used without abciximab, did not appear to improve renal function despite capturing debris. In the current study we saw a slight increase in platelet activation with the use of the EPD occurring immediately after the procedure, although this increase was not statistically significant. Conceivably the EPD may slow blood flow in the vessel, provide a surface upon which platelets can aggregate, and increase local platelet activation an effect inhibited by the GP IIb/IIIa inhibitor. Admittedly the observed increase in circulating levels of sCD40L with the use of the EPD was not statistically significant, however, it
may be unrealistic to expect that effects occurring on the surface of an EPD would be
detected systemically.

Several studies suggest a benefit of reducing platelet activation with loading doses
of 300-600 mg of clopidogrel prior to coronary interventions. However in the current
study pre-treatment with clopidogrel or clopidogrel administration on the day of
procedure did not significantly effect sCD40L levels. This may result from confounding
since patients were not randomized to clopidogrel treatment and had a significantly
higher prevalence of coronary artery and peripheral vascular disease, which may account
for the lack of difference observed in sCD40L levels. Work by Azar et al. reported a
reduction in sCD40L at a clopidogrel dose of 75 mg/day when preceded by a loading
dose of 300 mg in patients with stable CAD. Others though have failed to demonstrate
an effect of clopidogrel on levels of sCD40L.

Increased levels of circulating sCD40L and the impact on renal function in the
setting of RAS remain speculative. Future clinical trials should address the effect of
sCD40L inhibition on distal embolization and renal function with long term follow-up.
The current study provides a foundation for exploring the role of CD40/CD40L signaling
and the generation of renal fibrosis during ischemic renal injury.

The following limitations of our study warrant mentioning: The current study
utilized sCD40L as the key measure of platelet activation. We did not measure sCD40L
at one month nor do we have longer-term follow-up of renal function beyond one month.
Thus, it remains uncertain whether other indices of platelet activation would provide
additional insights, or whether longer term follow up would have yielded similar results for kidney function.

Atherosclerotic RAS is associated with increased platelet activation, but this increase appears to be attributable to atherosclerosis in general, not RAS specifically. However, in patients with higher levels of platelet activation prior to the procedure, embolization of platelet rich thrombi is more common. Abciximab effectively inhibits platelet activation and sCD40L release, a mechanism that may explain the beneficial effect on renal function one month after the procedure that has been previously observed.
4.6 Manuscript References


34. Nguyen TA, Lordkipanidze M, Diodati JG, Palaisaitis DA, Schampaert E, Turgeon J, Pharand C: Week-long high-maintenance dose clopidogrel regimen achieves


4.7 Table and Figure Legends

Table 4.1. Baseline Characteristics of the Normal Controls (Normal), Patient Controls (Control), and the Renal Artery Stenosis Patients (RAS). Values are mean ± SD, or number and percentage of patients. BMI, body mass index; sCD40L, soluble CD40 ligand; MI, myocardial infarction; NA, not applicable.

aNormal vs. RAS. bPatient control vs. RAS

Figure 4-1. Soluble CD40L levels in Normal Controls, Patient Controls, and Renal Artery Stenosis Patients (RAS). Box plot represents interquartile range with the median value shown as a horizontal bar within each box. Minimum and maximum values are shown in the bars outside each box. *p<0.001 vs Normal Control Subjects.

Figure 4-2. Soluble CD40L levels in patients With Platelet Rich Emboli Captured Within the filter at Immediate Post Procedure. Analysis of Angioguard contents was performed in 35/39 (90%) of patients randomized to Angioguard. Nine (9) out of 35 patients (26%) had platelet-rich emboli captured. Data presented as mean ± SEM, *p=0.02 vs Platelet Rich Emboli.

Figure 4-3. Soluble CD40L in patients with atherosclerotic renal artery stenosis randomly assigned to either abciximab, angioguard embolic protection, both, or neither. Data presented as mean ± SEM, *p<0.01 vs Baseline, #p<0.05 vs Baseline.
### Table 4.1.

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Normal (n = 30)</th>
<th>Control (n = 30)</th>
<th>RAS (n = 84)</th>
<th>P-Value*</th>
<th>P-Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>age (years) ± SD</td>
<td>35 ± 12</td>
<td>63 ± 12</td>
<td>73 ± 9</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
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<tr>
<td>female, n (%)</td>
<td>16 (53%)</td>
<td>17 (57%)</td>
<td>48 (57%)</td>
<td>0.72</td>
<td>0.96</td>
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<tr>
<td>white, non-Hispanic, n (%)</td>
<td>23 (77%)</td>
<td>26 (87%)</td>
<td>77 (92%)</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>BMI</td>
<td>25.9 ± 3.9</td>
<td>31.1 ± 8.7</td>
<td>28.6 ± 5.5</td>
<td>0.02</td>
<td>0.08</td>
</tr>
<tr>
<td>systolic BP (mmHg)</td>
<td>125 ± 2</td>
<td>126 ± 16</td>
<td>157 ± 31</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>diastolic BP (mmHg)</td>
<td>83 ± 3</td>
<td>78 ± 10</td>
<td>72 ± 16</td>
<td>&lt;0.001</td>
<td>0.13</td>
</tr>
<tr>
<td>heart rate</td>
<td>74 ± 3</td>
<td>65 ± 11</td>
<td>66 ± 12</td>
<td>&lt;0.001</td>
<td>0.96</td>
</tr>
<tr>
<td>Laboratory values</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>serum creatinine (mg/dl)</td>
<td>0.91 ± 0.10</td>
<td>0.85 ± 0.24</td>
<td>1.14 ± 0.41</td>
<td>0.003</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>sCD40L (pg/ml)</td>
<td>65.2 ± 7.4</td>
<td>335.2 ± 211.6</td>
<td>347.5 ± 248.3</td>
<td>&lt;0.001</td>
<td>0.81</td>
</tr>
<tr>
<td>platelet count (×1000)</td>
<td>298 ± 27</td>
<td>236 ± 54</td>
<td>239 ± 73</td>
<td>&lt;0.001</td>
<td>0.84</td>
</tr>
<tr>
<td>Indications for treatment, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>hypertension</td>
<td>NA</td>
<td>24 (80%)</td>
<td>84 (100%)</td>
<td>NA</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>congestive heart failure</td>
<td>NA</td>
<td>1 (3%)</td>
<td>20 (24%)</td>
<td>NA</td>
<td>0.01</td>
</tr>
<tr>
<td>renal dysfunction</td>
<td>NA</td>
<td>0 (0%)</td>
<td>18 (21%)</td>
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</tr>
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<td>angina</td>
<td>NA</td>
<td>21 (70%)</td>
<td>31 (37%)</td>
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<td>0.02</td>
</tr>
<tr>
<td>Risk factors, n (%)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>diabetes mellitus</td>
<td>NA</td>
<td>7 (23%)</td>
<td>21 (25%)</td>
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<td>0.86</td>
</tr>
<tr>
<td>peripheral vascular disease</td>
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<td>1 (3%)</td>
<td>43 (51%)</td>
<td>NA</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>hyperlipidemia</td>
<td>NA</td>
<td>22 (73%)</td>
<td>71 (85%)</td>
<td>NA</td>
<td>0.17</td>
</tr>
<tr>
<td>coronary artery disease</td>
<td>NA</td>
<td>13 (43%)</td>
<td>55 (65%)</td>
<td>NA</td>
<td>0.03</td>
</tr>
<tr>
<td>previous MI</td>
<td>NA</td>
<td>5 (17%)</td>
<td>26 (31%)</td>
<td>NA</td>
<td>0.13</td>
</tr>
<tr>
<td>history of smoking</td>
<td>NA</td>
<td>17 (57%)</td>
<td>53 (63%)</td>
<td>NA</td>
<td>0.53</td>
</tr>
<tr>
<td>Medications, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>antiplatelet</td>
<td>NA</td>
<td>20 (67%)</td>
<td>71 (85%)</td>
<td>NA</td>
<td>0.04</td>
</tr>
<tr>
<td>aspirin</td>
<td>NA</td>
<td>9 (30%)</td>
<td>39 (46%)</td>
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<td>0.12</td>
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<tr>
<td>thienopyridines</td>
<td>NA</td>
<td>2 (7%)</td>
<td>11 (13%)</td>
<td>NA</td>
<td>0.34</td>
</tr>
<tr>
<td>warfarin</td>
<td>NA</td>
<td></td>
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</tbody>
</table>
Figure 4-1.
Figure 4-2.
Figure 4-3
Chapter 5 - CD40 Mediated Fibrosis in Chronic and Ischemic Renal Disease

5.1 Chronic Kidney Disease and Renal Ischemia

Recent data indicates that chronic kidney disease (CKD) is prevalent, affecting up to 11% of the US adult population. Platelet activation and inflammation have been implicated in the progression CKD. Cardiovascular disease is both common and a major cause of mortality in patients with CKD. This uremic cardiomyopathy is characterized by a decrease in diastolic function, left ventricular hypertrophy, oxidant stress, and both cardiac and renal fibrosis. We have shown that the cardiotonic steroid marinobufagenin (MBG), signaling through the Na/K-ATPase, causes many of the adverse pathological effects of experimental uremic cardiomyopathy induced by 5/6th nephrectomy (PNx) in the rat. CTS bind to the Na/K-ATPase and convert it into a signal transducer capable of activating multiple protein kinase cascades. Sr"c binds to the Na/K-ATPase α1 subunit forming a functional signaling complex. Src transactivates EGFR which results in the activation of phospholipase C (PLC), phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinases (MAPKs), protein kinase C (PKC), and the generation of reactive oxygen species (ROS). We have demonstrated that pharmacologic administration of MBG causes cardiac hypertrophy and fibrosis, as seen in patients,
whereas active immunization against MBG attenuated this in PNx.\textsuperscript{7, 8} Additionally, cardiac fibroblasts treated with MBG, at concentrations similar to those reported in experimental and clinical renal failure, has been shown to stimulate collagen production.\textsuperscript{7} This increase in collagen production appears to be dependent on the Na/K-ATPase-Src-EGFR-ROS signaling cascade.\textsuperscript{7} The transcription factor Friend leukemia integration-1 (Fli-1) has been shown to be a negative regulator of collagen synthesis.\textsuperscript{13, 14} PKC-δ phosphorylates Fli-1 and promotes collagen synthesis.\textsuperscript{15} We have shown that MBG signaling through the Na/K-ATPase, cause PKC-δ translocation to the nucleus leading to Fli-1 phosphorylation and collagen production.\textsuperscript{16}

Renal artery stenosis (RAS) is a major cause of renal ischemia affecting 1-5% of the 60 million Americans with hypertension.\textsuperscript{17-19} Recent data suggests an incidence of up to 7% in patients over the age of 65.\textsuperscript{20} RAS is a major cause of secondary hypertension and an important cause of renal failure in patients with end stage renal disease.\textsuperscript{21-24} Although the clinical utility of stent revascularization in patients with RAS is still uncertain, several studies suggest that at least a portion of patients develop a loss of kidney function post-procedure.\textsuperscript{21, 22, 25, 26} Several mechanisms have been implicated as possible causes for a post-procedural decline in renal function such as contrast nephrotoxicity, and atheroembolization. Inflammation, fibrosis, and increases in oxidative stress leading to endothelial dysfunction have also been implicated in decreased renal function in the setting of atherosclerotic RAS.\textsuperscript{27} Increases in ROS have been reported in the stenotic kidneys from animal models of RAS.\textsuperscript{28, 29} We have shown that patients with RAS have significantly higher values of plasma MBG compared to healthy
control subjects, and patients with coronary atherosclerotic disease indicating that RAS potentiates MBG release.30

5.2 Platelet Activation, CD40 Signaling, and Fibrosis

Increased platelet activation is associated with a variety of vascular disorders including acute coronary syndromes, stable coronary artery disease, and restenosis following percutaneous coronary intervention.31, 32 Platelet activation leads to crucial integrin mediated signaling cascades, which result in stable interactions between platelets and the endothelium as well as activation of glycoprotein IIb/IIIa receptors (primary aggregation receptors), release of alpha and dense granules, and expression and secretion of sCD40L.33, 34 Soluble CD40 ligand has been shown to play a vital role in the immune, inflammatory, and coagulative responses following injury or stress, and implicated in the generation of renal fibrosis.35-39 Moreover, high levels of sCD40L correlate with adverse cardiovascular events in patients with unstable coronary syndromes, including atherothrombotic lesions.35, 40-42

CD40, a type-I transmembrane receptor and a member of the tumor necrosis factor (TNF) receptor superfamily 34, is expressed on a wide range of cells and critically links thrombosis, inflammation, immunity, and fibrosis. Recent work in renal disease models suggests that an important mediator of renal fibrosis and inflammatory cell infiltration is CD40 that resides on the surface of the proximal tubular epithelium. Specifically, CD40 is upregulated after renal injury 43 and activation of the receptor
results in 1) infiltration of inflammatory cells into the interstitium of the kidney through monocyte chemoattractant protein-1 (MCP-1), and intracellular adhesion molecule-1 (ICAM-1) expression, and 2) increases plasminogen activator inhibitor type 1 (PAI-1) and interstitial fibrosis. Importantly, Angiotensin II, whose release is increased during renal ischemia, increases TGF-ß that in turn markedly increases expression of CD40. Finally, CD40 activation increases antigen-specific recognition and killing of tubular epithelial cells by cytotoxic CD8+ T cells. Inhibition of CD40 significantly decreased the severity of renal injury in an animal model of chronic proteinuric renal disease. Our preliminary data shows a substantial increase in sCD40L in patients with renal artery stenosis compared to normal control subjects, as well as increased expression of CD40 in kidney tissue derived from PNx animals. We speculate that high levels of systemic sCD40L travel distally to the kidney and activate CD40 on the proximal tubules resulting in renal fibrosis.

5.3 Preliminary Data: Clinical trial

5.3.1 Soluble CD40 Ligand Levels in Patients with RAS

One hundred patients enrolled at 7 centers undergoing renal artery stenting were randomized to an embolic protection device (EPD), or double-blind use of a GPIIb/IIIa inhibitor, Abciximab (the RESIST trial). A detailed description of the clinical trial has been previously reported. Plasma levels of sCD40L were measured in all patients with available baseline blood samples (n=84) using a commercially available kit (R&D
Systems). Additional blood samples were collected from 30 healthy volunteers (Normal Controls), and 30 patients with atherosclerosis, but without renal artery stenosis (Patient Controls). A detailed description of this study has been reported and is described in Chapter 4.\textsuperscript{50} Platelet activation is an important component of the atherosclerotic process and shedding of sCD40L is a prominent feature of platelet activation. Our data confirms that normal healthy controls, free of atherosclerosis, have low levels of circulating sCD40L, whereas older subjects with atherosclerotic RAS are much higher (Figure 5-1).\textsuperscript{50} Importantly, the increase in sCD40L is not specific to renal artery stenosis, since a similar degree of elevation was observed in patients with atherosclerosis without RAS.

\textbf{Figure 5-1:} Soluble CD40 ligand levels in Normal Controls, Patient Controls, and RAS Patients. *p<0.01 vs. Normal Controls.
5.3.2 Circulating concentrations of marinobufagenin (MBG) are substantially increased in patients with ischemic renal disease.

Plasma MBG levels were measured in patients with ischemic renal disease obtained from the aforementioned “RESIST” trial, non-RAS patient controls who were scheduled for coronary angiography, and normal healthy individuals. Marinobufagenin levels were noted to be significantly higher in patients with RAS compared with those of the other 2 groups (Figure 5-2).\textsuperscript{30} Multivariate analysis shows that occurrence of RAS is independently related to marinobufagenin levels. In addition, renal artery revascularization by stenting partially reversed marinobufagenin levels in the patients with RAS.\textsuperscript{30}

![Figure 5-2: MBG levels in Normal Controls, Patient Controls, and RAS Patients.](image)

**Figure 5-2:** MBG levels in Normal Controls, Patient Controls, and RAS Patients.
5.3.3 Soluble CD40 concentrations appear to predict outcomes in patients with ischemic renal disease.

Very limited data is available on the effects of the CD40-sCD40L interactions on clinical outcome, with no published data in patients with ischemic RAS. Lajer et al reported higher levels of sCD40L in type-1 diabetics who developed nephropathy but no increase in mortality or rate of progression to ESRD.\textsuperscript{51} They had no data on CD40 and outcome. Recently we collaborated with Dr. Phillip Kalra in Manchester UK, assessing CD40 and sCD40L (measured at the University of Toledo) in a single center cohort of 126 patients with atherosclerotic RAS followed longitudinally. We have observed a statistically significant inverse correlation between baseline circulating levels of CD40 (not tissue bound) and change in GFR over time. Specifically, there was less loss of GFR (p=0.03) and better survival with higher baseline soluble CD40 (p=0.06). This is in agreement with the hypothesis that soluble CD40 “quenches” sCD40L thereby preventing activation of membrane-bound CD40. We also noted a trend toward increased mortality with higher levels of sCD40L (Figure 5-3).
Figure 5-3: Relationship between sCD40L and mortality rates in RAS patients. Note that in patients with high levels of sCD40L, mortality rates reach 40% during the follow up period obtained in the study. Because of the small numbers in this subset, the impressive trend is not statistically significant.

5.4 Preliminary Data: Animal Studies

We subjected Spargue-Dawley rats to either PNx surgery or infusion of MBG through minipumps at a dose designed to achieve similar elevations in plasma MBG as seen with PNx. We have previously demonstrated that this infusion of MBG produces substantial renal fibrosis by 4 weeks. When we examined the renal cortex of such animals (≥ 80% proximal tubule by volume), we noted substantial increases in CD40 expression (Figure 5-4, A). Of perhaps greater interest, we found that administration of either spironolactone or antibodies to MBG (either 3E9 mAb or digibind) resulted in marked decreases in renal fibrosis in the PNx model (Figure 5-5 and Figure 5-6, B).
work describing the use of 3E9 and digibind has recently been reported and is described in detail in Chapter 2.\textsuperscript{52} We also noted marked increases in signaling through CD40 as evidenced by PAI expression (Figure 5-4, B). We next observed that maneuvers which prevented MBG signaling through the Na/K-ATPase, either administration of antibody active against MBG or administration of the aldosterone antagonist, spironolactone, which we have also demonstrated directly inhibits CTS binding to the Na/K-ATPase\textsuperscript{53}, resulted in marked decreases in cortical CD40 expression (Figure 5-6, A, and Figure 5-7).

\textbf{Figure 5-4:} Representative Western blot and quantitative data of (A) CD40 and (B) PAI-1 expression derived from kidney cortex tissue, mean ± s.e.m. Sham (Sham operated controls); PNx (5/6\textsuperscript{th} nephrectomy); MBG (MBG infusion 10\(\mu\text{g/kg/day}\)). *p<0.01 vs. Sham, **p<0.05 vs. PNx.
**Figure 5-5:** Representative serius red fast green staining images (A), and (B) Western blot expression of collagen-1 with quantified data of kidney cortex tissue derived from Sham, PNx, Digibind (Dig), and 3E9 mAb treated animals, mean ± s.e.m. **p<0.01 vs. Sham, *p<0.05 vs. Sham, #p<0.01 vs. PNx, ##p<0.05 vs. PNx.
**Figure 5-6:** Representative Western blot and quantified data of (A) CD40, and (B) collagen-1 expression derived from kidney cortex tissue, mean ± s.e.m. Sham (Sham operated controls); PNx (5/6th nephrectomy); PNx+SP (PNx animals treated with spironolactone 80mg/kg/day); SP (Sham animals treated with spironolacton). **p<0.01 vs. Sham, *p<0.05 vs. Sham, #p<0.01 vs. PNx, ##p<0.01 vs. SP.
Figure 5-7: Representative Western blot and quantitative data of CD40 expression derived from kidney cortex tissue, mean ± s.e.m. Sham (Sham operated controls); PNx (5/6th nephrectomy); 3E9 (PNx animals treated with 3E9 mAb); Dig (PNx animals treated with Digibind). **p<0.01 vs. Sham, *p<0.05 vs. Sham, #p<0.01 vs. PNx, ##p<0.05 vs. PNx

5.5. Preliminary Data: LLC-PK1 cells

We have shown that MBG treatment in cardiac fibroblasts results in a substantial increase in procollagen-1 expression.\textsuperscript{7} Using a pig kidney proximal tubal cell line (LLC-PK1 cells), we have demonstrated that MBG treatment resulted in a significant increase in both CD40 and procollagen-1 expression (Figure 5-8, A and B). Reactive oxygen
species (ROS) have been shown to induce CD40 signaling in vascular smooth muscle cells.\textsuperscript{54} CTS signaling through the Na/K-ATPase induces ROS production and its downstream effects, such as cardiac and renal fibrosis, can be prevented by ROS scavenging.\textsuperscript{5} Our collaborators have developed an LLC-PK1 cell line in which the alpha 1 isoform of the Na/K-ATPase (required for CTS signaling) has been knocked down (PY-17 cells).\textsuperscript{55} Based on this background, we explored the potential crosstalk between MBG induced ROS and CD40 expression using the previously described cell lines as well as treatment with glucose oxidase (GO) which induces sustained levels of H\textsubscript{2}O\textsubscript{2}. MBG and GO treatment resulted in a significant increase in CD40 and procollagen-1 expression, and this effect was mitigated by treatment in PY-17 cells (Figures 5-9 and 5-10). Admittedly, the decrease in procollagen-1 expression in PY-17 cells is more pronounced than the decrease in CD40 expression. Our \textit{in vivo} results suggest that MBG signaling is required for procollagen-1 production, and contributes to increases in CD40 expression.
Figure 5-8: Representative Western blot and quantified data of CD40 expression (A) and procollagen-1 expression (B) from LLC-PK1 cells treated with MBG (10nM) for 24hrs (n=5 experiments). *<p<0.01 vs control
**Figure 5-9:** Representative Western blot and quantified data of CD40 expression from LLC-PK1 cells (LLC) and PY-17 cells treated with MBG (10nM) and GO (1 and 3 mU) for 24hrs (n=5 experiments). *p<0.01 vs. Controls, #p<0.05 vs. PY-17-MBG, PY-17-GO 1mU, and PY-17-GO 3mU
**Figure 5-10:** Representative Western blot and quantified data of procollagen-1 expression from LLC-PK1 cells (LLC) and PY-17 cells treated with MBG (10nM) and GO (1 and 3 mU) for 24hrs (n=5 experiments). *p<0.01 vs. Controls, #p<0.01 vs. PY-17-MBG, PY-17-GO 1mU, and PY-17-GO 3mU
5.6 Conclusions

The CD40/CD40L signaling cascade has been shown to induce inflammation and fibrosis in proximal tubular epithelial cells.\(^{39}\) Specifically, stimulation of the CD40 receptor by sCD40L causes increased expression MCP-1 and the pro-fibrotic mediator PAI-1 leading to the generation of fibrosis.\(^{39}\) Furthermore, inhibition of CD40/CD40L signaling has been shown to decrease the severity of renal injury in an animal model of chronic proteinuric renal disease.\(^{48}\) Our preliminary data from clinical trials demonstrates that 1) plasma levels of sCD40L and MBG are significantly increased in patients with ischemic renal disease, and 2) within these patients levels of circulation CD40 may predict renal function. Our data in animal models suggests that PNx and MBG administration results in significant increases in renal tissue expression of CD40 and PAI-1 whereas treatment with spironolactone, and antibodies to inhibit CTS signaling (digibind and 3E9) resulted in dramatic decreases in CD40 and collagen expression. Our future work will focus on expanding our preliminary results in patients with ischemic renal disease in order to determine if circulating levels of CD40 and CD40L are predictive of renal function. In addition, we will use genetic manipulation both \textit{in vivo} and \textit{in vitro} to knock out the CD40 receptor and knock down the Na/K-ATPase \(\alpha-1\) subunit. These manipulations will allow us to investigate the role of CTS signaling, CD40 signaling, and the generation of renal fibrosis. Our ultimate goal is to provide concrete evidence for our working hypothesis in the pathogenesis of renal fibrosis in the setting of chronic and ischemic renal disease (Figure 5-11).
Figure 5-11: Potential scheme for CD40 mediate renal fibrosis in the setting of chronic and ischemic renal disease.

In the setting of chronic and ischemic renal injury, increased circulating levels of MBG convert the Na/K-ATPase into a signal transducer generating ROS, which may lead to increased expression of CD40 resulting in pro-inflammatory and pro-fibrotic signaling cascades. CD40 signaling has also been implicated in the generation of ROS. Signaling through the CD40 receptor could be stimulated by high circulating levels of sCD40L. MBG signaling causes translocation of PKC-δ to the nucleus resulting in phosphorylation of FLI-1, and increased collagen expression leading to fibrosis.
5.7 References for Chapter 5


Chapter 6 – Summary and Conclusions

6.1 – Immunization As A Potential Therapy For Uremic Cardiomyopathy

Uremic cardiomyopathy is characterized as chronic renal failure accompanied with the development of severe cardiovascular disease, which ultimately accounts for the high morbidity and mortality in patients with this disease state. We have shown that the cardiotonic steroid, MBG signaling through the Na/K-ATPase significantly contributes to the development of experimental uremic cardiomyopathy induced by 5/6th nephrectomy (PNx) in the rat. Specifically, we have demonstrated that PNx animals develop diastolic dysfunction, cardiac hypertrophy, cardiac and renal fibrosis, elevated levels of cardiac and systemic oxidative stress, and elevated circulating levels of MBG. Chronic administration of MBG in normotensive rats (at a concentration similar to values reported in PNx animals) results in a similar cardiac phenotype as seen in PNx. We have demonstrated that active immunization against MBG in PNx animals attenuates these effects. In Chapter 2 we report that passive immunization with a single dose of an anti-MBG antibody (3E9 mAb) during the fifth week following PNx surgery drastically reduced systolic BP, cardiac fibrosis, and cardiac levels of oxidative stress. We also demonstrated that 3E9 treatment increased cardiac levels of Fli-1, a negative regulator of collagen synthesis. Our results indicate that immunization against MBG may provide a potential therapy for uremic cardiomyopathy.
6.2 – Treatment With Rapamycin As A Potential Therapy For Uremic Cardiomyopathy

The mTOR pathway has been implicated in the progression of many different forms of renal disease including experimentally induced uremic cardiomyopathy.\textsuperscript{4,5} Treatment with rapamycin (an mTOR inhibitor) has been shown to attenuate inflammation, fibrosis, and cardiac hypertrophy in experimental models of renal disease.\textsuperscript{4} In Chapter 3 we demonstrate that treatment with rapamycin in PNx animals significantly reduced cardiac fibrosis. Additionally, we were able to show that treatment with rapamycin in cardiac fibroblasts drastically reduced MBG induced collagen production when co-administered with MBG at concentrations similar to those reported in experimental and clinical renal failure.

The biosynthesis of MBG is currently under debate. In toads, MBG has been postulated to be synthesized from cholesterol via a bile acid pathway form cholic acids.\textsuperscript{1} In addition to acting as an mTOR inhibitor, rapamycin also acts as a competitive inhibitor of CYP27A1, a key rate-limiting enzyme of the bile acid pathway.\textsuperscript{6} Rapamycin treatment significantly reduced circulating levels of MBG in PNx animals. Treatment with rapamycin was also shown to drastically reduce MBG levels in human chorionic epithelial cells (JEG-3 cells), which produce MBG, by 52%. Our data demonstrates that rapamycin may offer a potential therapy for uremic cardiomyopathy acting as both an anti-fibrotic agent and a potential inhibitor of MBG production.
6.3 – Platelet Activation and CD40 Signaling in Chronic and Is ischemic Renal Disease

Increased platelet activation is associated with a variety of vascular disorders including acute coronary syndromes, stable coronary artery disease, and restenosis following percutaneous coronary intervention. Soluble CD40L is a particularly attractive marker for platelet activation since it is shed from the surface of activated platelets, is easily measured, and meaningfully participates in a number of important biologic processes including activation of immunity and thrombosis. In Chapter 4 we report that patients with renal artery stenosis have significantly high levels of circulating sCD40L compared to normal control subjects. However, this appears to be a non-specific association with atherosclerosis in general as opposed to being attributable to RAS specifically. More importantly though increased levels of sCD40L prior to the procedure were more likely to have embolization of platelet-rich thrombi and these patients had persistently elevated levels of sCD40L after the procedure. This finding may represent a potentially modifiable feature denoting increased risk for patients referred for renal artery revascularization.

CD40, a type-I transmembrane receptor and a member of the tumor necrosis factor (TNF) receptor superfamily, is expressed on a wide range of cells and critically links thrombosis, inflammation, immunity, and fibrosis. Recent work in renal disease models suggests that an important mediator of renal fibrosis and inflammatory cell infiltration is CD40 that resides on the surface of the proximal tubular epithelium. Specifically, stimulation of the CD40 receptor by sCD40L causes increased expression MCP-1 and the pro-fibrotic mediator PAI-1 leading to the generation of fibrosis. Our
preliminary data in animal models suggests that partial nephrectomy and MBG administration results in significantly increased expression of CD40 in the renal cortex tissue derived from these animals (Chapter 5). We also show increased expression of PAI-1 indicating an increase in CD40 signaling within the kidney (Chapter 5). Additionally, we provide evidence that maneuvers aimed at inhibiting cardiotonic steroid signaling through the Na/K-ATPase (spironolactone, 3E9, and digibind treatment) also resulted in a significant decrease in CD40 expression and renal fibrosis (Chapter 5). We speculate that in the setting of ischemic and chronic renal disease, increased circulating levels of sCD40L travel distally to the kidney, and activate the CD40 receptor resulting in the generation of renal fibrosis in a process potentiated by cardiotonic steroid signaling through the Na/K-ATPase. Future work will focus on determining a direct link between cardiotonic steroid signaling, CD40 signaling, and the development of renal fibrosis.
6.4 References for Summary and Conclusions


6. Gueguen Y, Ferrari L, Souidi M, Batt AM, Lutton C, Siest G, Visvikis S. Compared effect of immunosuppressive drugs cyclosporine A and rapamycin on...


