Cardiovascular complications of ischemic renal disease: the effect of renal dysfunction on cardiac disease and the central role of cardiotonic steroids in the pathogenesis of uremic cardiomyopathy

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Cardiovascular Complications of Ischemic Renal Disease: The Effect of Renal Dysfunction on Cardiac Disease and the Central Role of Cardiotonic Steroids in the Pathogenesis of Uremic Cardiomyopathy

Submitted by

David J. Kennedy

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

Date of Defense:

May 6, 2005

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DEDICATION

Totus tuus.
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INTRODUCTION

The care of patients with chronic renal failure is currently complicated by their propensity to develop severe abnormalities in cardiac physiological function as well as significant cardiac hypertrophy. This cardiac disease is directly responsible for much of the extremely high morbidity and mortality seen in chronic renal failure (Sarnak et al., 2003). The pathogenesis of this “uremic cardiomyopathy” is quite complex (1997; Foley et al., 1998; London and Parfrey, 1997; Middleton et al., 2001; Parfrey, 2001; Rigatto et al., 2002) and no effective clinical treatment strategy has yet been identified. There are many important issues relevant to the cardiovascular disease in this setting including hyperparathyroidism, hypertension, extracellular volume expansion and anemia. To be sure, the systemic oxidant stress state, marked cardiac hypertrophy and diastolic dysfunction, which accompany uremic cardiomyopathy, are still poorly understood. Interestingly, even mild degrees of chronic renal failure appear to confer a significant increase in cardiovascular disease (Garg et al., 2002; Henry et al., 2002).

On this background, it has been observed that steroid molecules, which bind to the plasmalemmal sodium potassium adenosine triphosphatase (Na/K-ATPase) and have structural similarity to the medication digitalis, accumulate in renal failure. These molecules have been referred to as digitalis-like substances or, more recently, cardiotonic steroids. Considerable effort has gone into the measurement of these molecules and the elucidation of their role in cardiac and renal physiology (de Wardener, 1996). Recent work has established that the cardiotonic steroid, marinobufagenin (MBG), induces
natriuresis and, in susceptible rat strains, increases blood pressure (Fedorova et al., 2002, 2005). Elevations of circulating MBG have been clearly demonstrated in both clinical and experimental renal failure whereas endogenous ouabain levels are not increased at 4 wk of experimental renal failure (Komiyama et al., 2005; Priyadarshi et al., 2003).

Our laboratory has observed that cardiotonic steroids, including those circulating in the serum of uremic patients, can acutely cause altered calcium cycling and cardiomyocyte relaxation through sodium pump inhibition (Periyasamy et al., 2001). Our group and others have observed that cardiotonic steroids induce signaling through the plasmalemmal Na/K-ATPase, which resides in caveolae (Liu et al., 2003, 2005). This signaling requires the generation of reactive oxygen species (ROS), has genomic effects that can be attributable to modulation of transcription factors including SP-1, and induces hypertrophic changes in both neonatal and adult cardiac myocytes in vitro (Haas et al., 2000, 2002; Huang et al., 1997; Kometiani et al., 1998; Liu et al., 2000; Xie et al., 1999). Recently, our group has shown that passive administration of antibodies raised against MBG reduced Na/K-ATPase endocytosis and sodium excretion in Sprague Dawley rats given a high salt diet (Periyasamy et al., 2005).

Therefore, based on data from our laboratory as well as the extensive literature concerning cardiotonic steroids, we propose the central hypothesis that circulating cardiotonic steroids bind to the Na/K-ATPase of cardiac myocytes and produce ROS as part of the resultant signaling cascade. This signaling cascade plays an essential role in
the pathogenesis of the diastolic dysfunction, cardiac hypertrophy as well as the systemic oxidant stress state seen in chronic renal failure.

In order to investigate this central hypothesis, we first investigated a clinical population of patients with ischemic nephropathy in order to examine the implications of stabilizing or improving renal function as it is related to the effects on cardiac morbidity and mortality. We then turned to the partial nephrectomy model in the rat, which has been used extensively to simulate experimental uremia in order to study the cardiac abnormalities, which accompany renal failure (Hostetter et al., 1981; Raine et al., 1993). Employing this model, we examined the molecular mechanisms by which chronic renal failure contributes to diastolic dysfunction and altered calcium handling. Next, we investigated the role that elevated levels of the cardiotonic steroid MBG plays in the cardiac abnormalities which accompany renal failure by testing if increases in circulating MBG were both necessary and sufficient to induce the systemic oxidant stress state, cardiac hypertrophy, and diastolic dysfunction seen with experimental uremia. Finally, using both isolated myocytes and an isolated perfused heart model, we endeavored to further examine novel ROS dependent mechanisms by which cardiotonic steroids might directly modulate both diastolic function and the physiologic activity of major calcium handling proteins.
LITERATURE

Epidemiology of Heart Disease in Chronic Renal Failure

Mortality Rates with Chronic Renal Failure

Hemodialysis represents a tremendous technical advance which allows a large number of patients who would otherwise expire from uremia to continue living. It is important to underscore, though, that hemodialysis does not effectively replace normal renal functions. Recent studies demonstrate that mortality rates in end stage renal disease (ESRD) patients remain extremely high in the United States. More than 50% of this mortality can be attributed to cardiac causes (1997; Sarnak et al., 2003). Conversely, it is not as if the high mortality seen with hemodialysis and peritoneal dialysis patients is simply due to complications of the therapies. Recent data strongly suggest that pre-ESRD patients have similar cardiac mortality rates as patients with ESRD (Paparello et al., 2002; Sarnak et al., 2003; Stack and Saran, 2002).

Frequency of Left Ventricular Hypertrophy in Chronic Renal Failure Patients

A number of studies utilizing echocardiography have demonstrated that both left ventricular hypertrophy (LVH) and diastolic dysfunction (as assessed by left ventricular, atrial and pulmonary venous doppler flow studies) are extremely common in ESRD patients treated with hemodialysis (Harnett et al., 1988) as well as patients incident to ESRD (Besarab and Aslam, 2001; Mitsnefes et al., 2001; Stack and Saran, 2002). In
general, most studies have demonstrated that LVH predicts diastolic dysfunction with some accuracy. Systolic dysfunction, while not uncommon, is less often demonstrable than diastolic dysfunction and LVH (Raj et al., 1997). Parfrey and colleagues have demonstrated that the development of LVH in hemodialysis patients predicts a high mortality rate (Parfrey, 1996, 2001). Others point out a correlation between the degree of LVH as assessed by left ventricular mass index (LVMI) and the occurrence of ventricular arrhythmias in hemodialysis patients (Nakazato et al., 2002; Tyralla and Amann, 2003; Vasan et al., 1997). In short, ESRD patients treated with hemodialysis have a high prevalence of LVH, and the clinical implications of LVH in this population are grave.

There are epidemiological data which support the concept that hemodialysis contributes to the development of LVH. Raj and coworkers compared a group of ESRD patients not yet receiving hemodialysis to a group of ESRD patients receiving dialysis as well as normal controls. The nondialized ESRD patients were not found to have LVH in greater frequency than controls. However, the ESRD patients treated with hemodialysis had a very high prevalence of LVH (Raj et al., 1997). Greaves and coworkers, found only a slightly higher prevalence of LVH in ESRD patients treated with hemodialysis, but many of the pre-ESRD patients were hypertensive (Greaves et al., 1994). De Lima and coworkers demonstrated that while normotensive ESRD patients treated with hemodialysis had a comparable LVMI to nonuremic controls, hypertensive ESRD patients treated with hemodialysis had a markedly higher LVMI as compared with nonuremic patients with comparable increases in blood pressure (De Lima et al., 1992).
Pathophysiology of Cardiac Hypertrophy in Chronic Renal Failure

Anemia

Anemia commonly complicates chronic renal failure, and is essentially the rule in ESRD patients treated with intermittent hemodialysis. The best data to support a pathophysiological role for anemia in the LVH of ESRD patients is the observation that treatment with recombinant erythropoietin may actually cause regression in LVH to some degree (Frank et al., 2004; Hassan et al., 2005; Hayashi et al., 2000; Sunder-Plassmann and Horl, 2001). In some studies, a 10-20% reduction in LVMI has been observed with sustained increases in hematocrit (Kausz et al., 2000). In contrast, the type of hypertrophy observed in ESRD patients is usually of the concentric type; this is not the hypertrophy pattern that one would expect if anemia were the dominant factor. Also, multiple regression analyses have generally found only a relatively small correlation between hemoglobin (or hematocrit) and LVH (Erten et al., 2005; Neves et al., 1997; Tell et al., 1994). Probably because of worsened blood pressure control, Minagawa and colleagues found that erythropoietin therapy actually worsened LVH (Minagawa et al., 1994).
Hypertension

Hypertension is the physiological factor in ESRD patients that has been best linked to LVH. A number of publications demonstrate significant correlations between the magnitude of LVH on echocardiogram and either the predialysis blood pressure, 24-hour ambulatory blood pressure or number of antihypertensive medications (Harnett et al., 1994; Morduchowicz et al., 1993; Ness et al., 1999; Raj et al., 1997). Certainly hypertension is a treatable factor that must be addressed aggressively in patients with ESRD. However, it is important to note that while hypertension certainly contributes to the LVH seen in ESRD, blood pressure alone cannot explain the frequency and severity of LVH in ESRD patients (De Lima et al., 1992; Harnett et al., 1995; Huting and Alpert, 1992; Nishikimi et al., 2001).

Parathyroid Hormone

Hyperparathyroidism causes abnormalities in cardiac energy metabolism, function and growth in experimental and clinical settings (Amann and Ritz, 1997; Foley et al., 1996; London and Parfrey, 1997; Parfrey and Harnett, 1994 a). On a molecular basis, it is possible (although not studied to date) that parathyroid hormone stimulated Na/K-ATPase endocytosis (Khundmiri et al., 2004) might actually amplify cardiotonic steroid signaling through the Na/K-ATPase.
Hyperparathyroidism also has been associated with LVH in several clinical studies of patients with renal failure, including regression of LVH in patients with ESRD following parathyroidectomy (Amann and Ritz, 1997; Covic et al., 1996; Foley et al., 1996; London and Parfrey, 1997). However, it is clear that significant hyperparathyroidism is not a necessary condition for the development of LVH in ESRD patients (Harnett et al., 1994; Ifudu et al., 1998).

Other Factors

In addition to the aforementioned pathogenic factors, the renin angiotensin system (Cannella, 1996; Cannella et al., 1997; Dyadyk et al., 1997; Vlahakos et al., 1997) as well as lipid abnormalities (London and Parfrey, 1997) have been suggested to play a role in the pathogenesis of LVH in ESRD patients treated with hemodialysis. In addition, smaller studies have identified other factors ranging from aluminum intoxication to activation of the sympathetic nervous system (Bernardi et al., 1984; London et al., 1989).

Excitation-Contraction Coupling

The cardiac action potential elicits contraction of myocytes through depolarization and activation of Ca\(^{2+}\) channels (mainly L-type) that create an inward Ca\(^{2+}\) current (I\(_{Ca}\)). This Ca\(^{2+}\) influx initiates Ca\(^{2+}\)-induced-Ca\(^{2+}\)-release from the sarcoplasmic reticulum whereby local Ca\(^{2+}\) entry increases the probability of opening of a proximate RyR (Eisner et al.,
This causes increases in free [Ca\(^{2+}\)]\(_i\) and increased binding of cytosolic Ca\(^{2+}\) buffers (e.g., Troponin C). Binding of Ca\(^{2+}\) to TnC switches on the myofilaments and synergistically activates contraction. For relaxation, Ca\(^{2+}\) is removed from the cytosol through four critical pathways: 1) Sarcoplasmic reticulum calcium ATPase (SERCA), 2) sarcolemmal sodium calcium exchanger (NCX), 3) sarcolemmal Ca\(^{2+}\)-ATPase, and 4) mitochondrial Ca\(^{2+}\) uniporter. Quantitatively, SERCA and NCX account for the vast majority of Ca\(^{2+}\) removal under normal conditions (Bers, 2002). In heart failure, alterations in cardiac Ca\(^{2+}\) handling are the foundation of the observed pathophysiologic contractility and Ca\(^{2+}\) transients, however, the molecular changes that occur and their relative contributions to Ca\(^{2+}\) handling are controversial (Eisner and Trafford, 2002). Several lines of evidence, however, implicate SERCA2a as a critical determinant of cardiac diastolic function (Lehnart et al., 1998; Mercadier et al., 1990; Schmidt et al., 1998; Schotten et al., 1999).
SERCA Biology

Three distinct SERCA genes (SERCA 1, 2, and 3) have been cloned to date (Lytton and MacLennan, 1988; Lytton et al., 1989; MacLennan et al., 1985). The SERCA1 gene contains alternatively spliced transcripts SERCA1a and SERCA1b, and is expressed in fast-twitch skeletal muscle (MacLennan et al., 1985). The SERCA2 gene encodes SERCA2a and SERCA2b which differ in their carboxy terminus and 3’ untranslated region (Lytton and MacLennan, 1988; Misquitta et al., 2002). SERCA2a is highly expressed in cardiac and slow-twitch muscle (Anger et al., 1994) and is believed to play the greatest role in cardiac Ca^{2+} cycling. SERCA2b is rather ubiquitous (Lytton et al., 1989) while the SERCA3 gene is expressed in epithelial and endothelial cells as well as those of hematopoietic origin (Anger et al., 1993). Notably, SERCA isoforms appear to exhibit varying sensitivity to ROS, and SERCA2 appears to be quite sensitive to both superoxide and peroxide (Barnes et al., 2000; Grover and Samson, 1997; Grover et al., 1997).

Sodium Pump Biology

Na/K-ATPase was discovered by Skou in 1957 (Skou, 1957). Since then work from numerous laboratories (Lingrel et al., 1994 a, b; Schwartz et al., 1988; Sweedner, 1989) established the following conclusions: (a) This enzyme is indeed the molecular machine for the ATP dependent and coupled transports of Na^+ and K^+ across the plasma...
membranes of all cells in which such a process (sodium pump) is detected; (b) Cardiac Na/K-ATPase is the functional receptor for the inotropic effects of digitalis. Inhibition of the enzyme by digitalis in the heart raises [Ca$^{2+}$]$_i$ and myocardial contractility. This serves as the basis for the use of digitalis drugs in the therapy of congestive heart failure. On this foundation, cumulative studies from our laboratory and others in recent years show that the enzyme also is a signal transducer involved in regulation of multiple gene-regulatory second messengers and pathways including activation of the signaling molecules v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog, epidermal growth factor receptor (EGFR), Ras, protein kinase C, mitogen-activated protein kinase (MAPK), and intracellular ROS production (Aizman et al., 2001; Aydemir-Koksoy et al., 2001; Aydemir-Koksoy and Allen, 2001; Belusa et al., 2002; Haas et al., 2000, 2002; Huang et al., 1997; Kometiani et al., 1998, 2001; Liu et al., 2000, 2002; Mohammadi et al., 2001; Peng et al., 1996; Xie, 2001; Xie and Askari, 2002; Xie et al., 1999).

**Structure of the Na/K-ATPase and SERCA**

The Na/K-ATPase belongs to the family of P-type ATPases and consists of two noncovalently linked $\alpha$ and $\beta$ subunits (Lingrel et al., 1994 a, b; Sweedner, 1989). The $\alpha$ subunit (about 112 kDa) contains the ATP, digitalis, and other ligand binding sites, and is considered as the “catalytic subunit.” The $\alpha$ subunit is essential for the assembly of the functional enzyme. Several $\alpha$ and $\beta$ subunits have been identified and functionally characterized (Lingrel et al., 1994 a, b; Sweedner, 1989). The isoforms are expressed in a
tissue-specific manner. The $\alpha_1$ isoform is found in all cells whereas the $\alpha_2$ and $\alpha_3$ isoforms are expressed in skeletal muscle, neuronal tissue, and cardiac myocytes (Lingrel et al., 1988, 1990). Recently, the crystal structure of SERCA1a has been determined at 2.6 Å resolution (Toyoshima et al., 2000). Both SERCA and the Na/K-ATPase belong to the type-II class of P-type ATPases, and both contain four distinct functional domains (Sweedner and Donnet, 2001). The actuator (A) domain consists of the N-terminus and the first cytoplasmic loop connected to transmembrane helices M2 and M3. Although there is great sequence variation at the N-terminus between SERCA and the Na/K-ATPase, both enzymes appear to have the same two $\alpha$-helix motifs. Most importantly, based on the structure of SERCA, the A domain is highly exposed for binding of other proteins. The enzyme also has the highly conserved phosphorylation (P) domain that is close to the membrane and a relatively isolated nucleotide binding (N) domain (Sweedner and Donnet, 2001).

**SERCA and $\text{Ca}^{2+}$ Homeostasis**

The role of SERCA2a in myocardial $\text{Ca}^{2+}$ removal is varied across species. In rabbit, dog and human ventricles, SERCA2a represents roughly 70% of $\text{Ca}^{2+}$ removal while NCX accounts for the bulk of the remainder (Bassani et al., 1994; Bers, 2002). Alternatively, because of the greater concentration of pumps, rat and mouse SERCA2a represents >90% of $\text{Ca}^{2+}$ removal during diastole (Bers, 2002; Hove-Madsen and Bers, 1993). In all species, the activity of SERCA is reduced by the accessory protein phospholamban
(PLB) and phosphorylation of PLB removes this inhibition (Koss and Kranias, 1996). This provides a mechanism whereby sympathetic stimulation, via protein kinase A (PKA) or calcium-calmodulin kinase II mediated phosphorylation, can increase sarcoplasmic reticulum Ca\(^{2+}\) content and thus Ca\(^{2+}\) release from the sarcoplasmic reticulum. Notably, by decreasing SERCA activity and therefore sarcoplasmic reticulum Ca\(^{2+}\) content, the amplitude and rate of decay for the Ca\(^{2+}\) transient may be depressed.

**Ca\(^{2+}\) Homeostasis in Heart Failure**

In the failing heart, there is a debate about the Ca\(^{2+}\) handling mechanisms responsible for the systolic and diastolic dysfunction. There is widespread acceptance of the phenomenon of decreased sarcoplasmic reticulum Ca\(^{2+}\) release in heart failure, most likely owing to a reduction in sarcoplasmic reticulum Ca\(^{2+}\) content (Paparello et al., 2002; Pogwizd et al., 2001). However, whether such a decrease is due to changes in either the RyR, SERCA2a, or a combination of the two is a topic of controversy (Eisner and Trafford, 2002).

The sarcoplasmic reticulum Ca\(^{2+}\) release could be affected by functional changes in the RyR or its sensitivity to I\(_{Ca}\), whereby fewer RyR would open for a given I\(_{Ca}\) stimulus. The RyR can be regulated by PKA phosphorylation and by the accessory protein FK-506 binding protein 12 (FKBP12). Thus, PKA mediated phosphorylation may increase the open probability of the RyR (Takasago et al., 1989), while FKBP12 stabilizes interactions between RyRs allowing coupled gating and release of adjacent channels.
Disruption of FKBP12 from RyR could potentially cause unstable channels which leak Ca\(^{2+}\) (thereby decreasing sarcoplasmic reticulum Ca\(^{2+}\) load) (Xiao et al., 1997). It has thus been postulated that rising catecholamine levels in the setting of heart failure, cause a PKA mediated disruption of the RyR/FKBP12 complex, and therefore, cause decreased sarcoplasmic reticulum Ca\(^{2+}\) load by increasing RyR leak (Marx et al., 2000). This observation has been used to explain the efficacy of β-blockers (via reduced RyR phosphorylation) in heart failure (Marx et al., 2000; Reiken et al., 2001) although this finding has been challenged recently (Jiang et al., 2002; Li et al., 2002).

Nevertheless, decreases in SERCA2a activity routinely complicate experimental heart failure (Lehnart et al., 1998; Mercadier et al., 1990; Schmidt et al., 1998; Schotten et al., 1999). Several novel gene delivery strategies have given support for this hypothesis. In vivo cardiac recombinant adeno-associated virus expressing a pseudophosphorylated mutant of PLB in a hamster model of dilated cardiomyopathy has been used to enhance myocardial sarcoplasmic reticulum Ca\(^{2+}\) uptake which resulted in suppression of progressively impaired left ventricular systolic function, pressure, contractility, and left ventricular relaxation (Hoshijima et al., 2002). Hajjar and colleagues have demonstrated improvement in systolic, diastolic and contractile function (del Monte et al., 1999), along with improved cardiac energetics and survival (del Monte et al., 2001) by SERCA2a overexpression in a pressure overload (aortic clip) model of heart failure. Periasamy and colleagues used a similar approach with SERCA1a overexpression to demonstrate that
this isoform may substitute structurally and functionally for SERCA2a while demonstrating faster Ca\(^{2+}\) handling kinetics as well as faster rates of contraction and relaxation (Lalli et al., 2001; Sells et al., 1995).

**Circulating Cardiotonic Steroids in Mammalian Physiology and Chronic Renal Failure**

One of the neurohumoral systems which has attracted enormous attention from the nephrology community is the substance(s) initially referred to as “3rd factor” (Bricker, 1967). Over the years, a number of workers such as deWardener suggested that “3rd factor” is an inhibitor of the sodium pump (de Wardener, 1996). In the late 70s and early 80s, it became clear that patients with chronic renal failure often had “false positive” digoxin levels (i.e., digoxin was detected when the patient was not taking this medication) with some antibodies (Graves, 1986; Graves and Williams, 1987). Increases in the circulating concentrations of digitalis-like sodium pump ligands has been postulated for some time in chronic renal failure as an adaptive response to volume expansion, whereby volume may be decreased by inhibition of the Na/K ATPase of the renal tubules (Blaustein, 1993; de Wardener and Clarkson, 1985). In addition to playing a role in sodium balance, Bricker and others proposed that effects of cardiotonic steroids on other tissues explained aspects of the uremic syndrome which complicates chronic renal failure. The term “trade off hypothesis” was coined to explain this phenomenon (Bricker and Fine, 1978).
Our understanding of this issue has evolved considerably. It now appears that ouabain, the prototypical digitalis glycoside (or something structurally quite similar), is essentially a neurohormone, and that MBG (or something structurally quite similar) is the circulating cardiotonic steroid which has a substantial effect on the α1 isoform of the Na/K-ATPase. MBG concentrations in the plasma increase in a variety of experimental and clinical settings associated with volume expansion and hypertension (Fedorova et al., 1998 a, b; Gonick et al., 1998; Lopatin et al., 1999). Recently, it has been demonstrated that synthesis of this substance occurs in mammalian adrenal cells (Dmitrieva et al., 2000). Interestingly, modulation of the response of Na/K-ATPase to MBG appears to result from PKC inhibition (Bagrov et al., 2000). It appears that an initial transient stimulation of brain derived ouabain or ouabain-like compounds induced by salt loading precedes a sustained MBG response and contributes to the chronic blood pressure elevation in Dahl salt sensitive rats (Fedorova et al., 2002). This initial increase of a ouabain-like compound agrees with the results of prior experiments in which the importance of brain ouabain in the onset of NaCl-induced hypertension has been noted where the blockade of brain ouabain with digoxin antibody has been shown to alleviate NaCl-induced hypertension in Dahl salt sensitive rats (Abdelrahman et al., 1995; Huang and Leenen, 1994). The regulation of MBG summarized from the aforementioned studies is displayed in Figure 1.
Our recent understanding of this area has increased because of a synthesis of basic science with clinical physiology. For years, concerns were voiced as to how circulating hormone concentrations in the high picomolar to low nanomolar range could be meaningful participants in physiology when the Na/K-ATPase isoforms all had IC50 values for these hormones in the micromolar range. However, a number of laboratories have demonstrated that very low levels of MBG and ouabain can have very meaningful physiological effects. The laboratory of JC Allen has shown that picomolar concentrations of ouabain and cardiotonic steroids have proliferative effects on smooth muscle cells derived from humans and rodents (Abramowitz et al., 2003; Allen et al., 2003). Doris also has shown growth effects of these concentrations in epithelial cells derived from rats where the actual Na/K-ATPase enzymatic inhibition would simply not be detectable (Dmitrieva and Doris, 2003). Our group also has demonstrated that low concentrations of ouabain and MBG can induce demonstrable increases in the growth of adult rat cardiac myocytes grown in culture (Priyadarshi et al., 2003). We also have
observed that very low concentrations of these hormones induce signal transduction events including Na/K-ATPase endocytosis in LLC-PK1 cells (Liu et al., 2002). Perhaps of greatest relevance is the recent, seminal paper from the laboratory of Bianchi where it was clearly demonstrated that infusion of 15 ug/kg/day of ouabain produced a syndrome of hypertension and left ventricular hypertrophy in Sprague Dawley rats. This dose of ouabain resulted in an approximate two to three times elevation of baseline ouabain or ouabain-like compound concentrations determined with an immunoassay. These investigators went on to define a caveolar compartment of the plasmalemmal Na/K-ATPase where small amounts of ouabain apparently had substantial physiological effects as well as demonstrate amelioration of this effect of ouabain by an experimental cardiac steroid, PS2238 which apparently binds to the Na/K-ATPase without initiating signal transduction (Ferrandi et al., 2004). In short, the long term effects of cardiotonic steroids on cell biological processes including hypertrophy and hyperplasia do not appear to require substantial inhibition of the plasmalemmal Na/K-ATPase.

Of the circulating cardiotonic steroids that have been identified, ouabain has perhaps been the best characterized. Ouabain is a cardiac steroid derived from plant tissue, and it is ouabain that is probably the cardiotonic steroid of first choice for study in laboratory preparations. As discussed above, a compound that is immunologically quite similar to plant derived ouabain can be detected in a number of mammalian tissues. Recent studies have isolated such an ouabain-like compound from the hypothalamus of cattle, and identified this ouabain molecule to be an optical isomer of ouabain derived from plants.
Artifacts determined during the isolation procedure require that further work remains to for unequivocal determination of the chemical structure of ouabain-like compound (Kawamura et al., 1999). Bufalin and MBG also have been detected in body fluids with Ab based assays. Studies from the laboratory of Bagrov have identified an Ab assay capable of recognizing a chemical with the same retention time on a high performance liquid chromatograph column and same mass determined with mass spectroscopy as amphibian derived MBG (Bagrov and Fedorova, 1998). Perhaps the most convincing identification was performed by Komiyama and colleagues who identified MBG and a derivative, telocinobufagin, in human plasma of ESRD patients using both high-resolution mass spectrometry and nuclear magnetic resonance (Komiyama et al., 2005). The chemical structures of both ouabain and marinobufagenin are shown in Figures 2 and 3.

Figure 2. Chemical Structure of Ouabain
Data will be reviewed below that some signaling through the sodium pump involves the generation of ROS (Liu et al., 2000). However, it also appears that ROS may initiate signaling through this system by inhibiting NaK-ATPase function creating a potential positive feed-back loop (Xie et al., 1995). Since both ROS and cardiotonic steroids are increased in chronic renal failure patients, we believe that interaction between ROS and other circulating pump inhibitors can cause a significant inhibition of the enzyme, resulting in activation of multiple pathways of cardiac hypertrophy (Xie et al., 1999) including downregulation of SERCA2a (Kennedy et al., 2003). This certainly represents an important risk factor for development of cardiac hypertrophy and diastolic dysfunction of the heart in chronic renal failure patients.
The Na/K ATPase as a Signal Transducer

Na/K-ATPase as an energy transducing ion pump has been studied extensively since its discovery in 1957 (Skou, 1957). Although early findings suggested that the enzyme also plays a role in regulation of gene expression and cell growth (Nakagawa et al., 1992; Pressley, 1992) only in recent years have the signal transduction mechanisms associated with sodium pump inhibition been investigated. This research, done mostly on rat cardiac myocytes, shows that in addition to pumping ions, Na/K-ATPase interacts with neighboring membrane proteins and organized cytosolic cascades of signaling complexes to send messages to various intracellular organelles (Liu et al., 2000). The down-stream events related to this signal transduction includes increases in [Ca$^{2+}$], and contractility as well as changes in the expression of a number of cardiac growth-related genes including ANP and skACT and stimulation of protein synthesis and myocyte hypertrophy (Kometiani et al., 1998; Liu et al., 2000; Peng et al., 1996; Xie et al., 1999). We have recently demonstrated that the sodium pump inhibitors present in the serum of patients with chronic renal failure produce the same physiological and biochemical effects in adult rat cardiac myocytes as ouabain (Periyasamy et al., 2001).

Additionally, we have demonstrated in both neonatal and adult cardiac myocytes that ouabain binding to Na/K ATPase activates Src kinase which transactivates EGFR, resulting in the activation of the Ras/Raf/MEK/MAPK cascade (Haas et al., 2000; Tian et al., 2001). This activation of p42/44 MAPKs amplifies the ouabain-induced increases in
Importantly, increases in $[\text{Ca}^{2+}]_i$ can further activate p42/44 MAPKs (Bogoyevitch et al., 1996; Sadoshima et al., 1995). Furthermore, increases in ROS and activation of p42/44 MAPKs have been demonstrated to increase Egr-1, an important transcription factor responsible for downregulation of SERCA2 gene expression in neonatal myocytes (Arai et al., 2000). It is in this context that we propose ROS as mediators for the decreases in the expression and activity of SERCA2a and that such changes are exacerbated by cardiotonic steroid signaling. This hypothesis was generated from both the data summarized above and as well as a recent report from our laboratory that cardiomyocytes from uremic rats ($5/6^{th}$ Nephrectomy) exhibit increased production of ROS which stimulate hypertrophic growth and inhibit Na/K ATPase expression and activity – effects which were blocked by antioxidant therapy (Priyadarshi et al., 2003). This relationship among cardiotonic steroids, ROS, Na/K ATPase and regulation of SERCA2a is shown in Figure 4.
This schematic is supported by data from the chronic renal failure model that demonstrates decreases in the expression and activity of SERCA2a and the Na/K ATPase, elevated levels of cardiotonic steroids (Ouabain-like, MBG) and increased levels of ROS. Binding of cardiotonic steroids to the Na/K ATPase initiates a signal cascade beginning with tyrosine phosphorylation of Src which leads to activation of Ras. Following Ras activation, the signal apparently diverges with the activation of MAPK not requiring ROS but the genomic/hypertrophic gene regulatory events appear to involve intracellularly generated ROS as essential second messengers. Anti-oxidants appear to block the ouabain induced inhibition of the Na/K ATPase. We propose that ROS may modulate SERCA2a expression and/or activity either by direct oxidation of susceptible residues or indirectly by effecting the expression of transcription factors with putative binding elements in the SERCA2a promoter region (e.g. SP-1, Egr-1). These data are summarized in references (Adachi et al., 2004; Arai et al., 2000; Huang et al., 1997 a, b; Kennedy et al., 2003; Koitabashi et al., 2005; Kometiani et al., 1998; Liu et al., 2000; Peng et al., 1995; Priyadarshi et al., 2003; Xie et al., 1999, 2000).
Significantly, many of the above findings also have been reported recently in cells other than cardiac myocytes. Binding of ouabain to the Na/K-ATPase stimulates Src, EGFR, calcium oscillation and MAPKs in several different cells including smooth muscle cells and kidney tubular cells (Aizman et al., 2001; Aydemir-Koksoy et al., 2001; Aydemir-Koksoy and Allen, 2001; Aydemir-Koksoy and Allen, 2001; Haas et al., 2000; Haas et al., 2002).

Because many of the signaling events are activated by ouabain in the absence of changes in intracellular ion concentrations (Aizman et al., 2001; Aydemir-Koksoy et al., 2001; Aydemir-Koksoy and Allen, 2001 a, b; Tian et al., 2001), we have recently proposed that the ouabain-bound (activated) Na/K-ATPase is capable of recruiting and activating protein tyrosine kinases through specific protein-protein interactions (Xie and Askari, 2002). Realization that the Na/K-ATPase is a signal transducer has prompted us to examine the potential ligands for the Na/K-ATPase which might stimulate activation of the signal transduction pathway. In principle, there are at least three major potential classes of chemicals that could serve as the Na/K-ATPase ligands under both physiological and pathological conditions. The first group of chemicals include those that bind to and reversibly inhibit the Na/K-ATPase, such as the cardiotonic steroids (e.g., endogenous ouabain or MBG like factors) (Fedorova et al., 2001; Hamlyn et al., 1991). The second class includes those that chemically modify the enzyme either reversibly or irreversibly. It appears that ROS fit into this category (Xie et al., 1990, 1999). The third group are not chemicals per se, but rather alterations in the concentrations of intracellular...
sodium and extracellular potassium. We have observed that decreases in extracellular potassium not only potentiates the effects of cardiac steroids but also induces signaling through the Na/K-ATPase by itself (Xie et al., 2000).

**Reactive Oxygen Species, Na/K ATPase, SERCA2a, and Cardiovascular Disease**

Recent studies have demonstrated that ROS mediate a number of physiological processes (Irani et al., 1997; Yeh et al., 1999) in addition to their well characterized role in pathological conditions such as ischemia-reflow (McCord, 1985; McCord and Fridovich, 1978). We have previously shown that the signal cascade resulting from sodium pump inhibition has an absolute requirement for ROS in order to affect gene transcription of ANP and skACT and hypertrophy in cardiac myocytes (Liu et al., 2000; Xie et al., 1999).

Oxidant stress has been postulated to play a role in the progression of chronic renal failure since the late 1980s (Nath and Paller, 1990; Shapiro, 1990) including linkage between oxidant stress in hemodialysis patients and cardiovascular disease (Galli et al., 1999; Smith and Berkseth, 1990; Spittle et al., 2001; Tetta et al., 1999; Usberti et al., 2002). As noted previously, it also appears that ROS may also inhibit the Na/K-ATPase and initiate its signaling function, thus creating a potential positive feed-back loop (Xie et al., 1995).
There are several ways in which ROS may make important contributions to the pathophysiology of heart failure. Mechanisms such as the initiation of myocyte apoptosis through nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 or by contributing to endothelial dysfunction by reducing the bioavailability of nitric oxide and regulating endothelial nitric oxide synthase mRNA stability have been proposed (Lopez Farre and Casado, 2001). The ROS also can affect a variety of other cellular processes (Abe and Berk, 1998; Abe et al., 2000; Suzuki et al., 1997). As signaling molecules they can activate tyrosine kinases, interact with the Ras signaling network, regulate intracellular Ca\(^{2+}\) and modulate activities of transcription factors such as AP-1 and Egr-1. Significantly, ROS activation of Src family kinases plays an important role in ROS signaling (Abe et al., 2000; Cai and Jones, 1998; Chen et al., 2001; Sabri et al., 1998). For example, exposure of endothelial cells to H\(_2\)O\(_2\) was found to transactivate EGFR, and subsequently stimulate JNK and p42/44 MAPK cascades (Chen et al., 2001). Inhibition of Src kinase abolished H\(_2\)O\(_2\)-induced transactivation of EGFR and stimulation of both JNK and p42/44 MAPKs and also caused a significant inhibition of the Na/K-ATPase. Of particular importance, however, is the finding that ROS may exert direct negatively ionotropic effects by depressing sarcoplasmic reticulum calcium uptake and reducing SERCA2 activity (Rowe et al., 1983).
Regulation of Reactive Oxygen Species

The major ROS responsible for oxidative stress in heart failure are $\mathrm{O}_2^-$, $\mathrm{H}_2\mathrm{O}_2$, and $\mathrm{OH}^\cdot$. $\mathrm{O}_2^-$ is generated mainly from mitochondrial oxidation and enzymatic sources including nicotinamide adenine dinucleotide phosphate oxidase, xanthine oxidase, and the endothelial nitric oxide synthase enzyme (Lopez Farre and Casado, 2001). Cellular defense mechanisms also exist against oxidative stress such as superoxide dismutase, catalase, and glutathione peroxidase (Fridovich, 1997; Yu, 1994). In the heart, mitochondrial and cytoplasmic superoxide dismutase catalyzes the dismutation of $\mathrm{O}_2^-$ into $\mathrm{H}_2\mathrm{O}_2$ and oxygen. Next, catalase (present in peroxisomes) and glutathione peroxidase (in the cytoplasm) hydrolyze the $\mathrm{H}_2\mathrm{O}_2$ generated from this reaction. Albumin, along with iron binding and transport proteins function as extracellular antioxidants by lowering the free iron concentrations that can promote lipid peroxidation. Water soluble Vitamin C, NAC, and GT also can act extracellularly to prevent lipid peroxidation, while lipid soluble vitamin E does so intracellularly.

Reactive Oxygen Species and SERCA2a mRNA Stability

It is possible that ROS could act post-transcriptionally but pre-translationally to decrease SERCA2a mRNA half-life and thus expression. In fact, some mRNA’s contain sequences located in the 3’ untranslated region which regulate its stability. In this region, specific sequences (cis-acting or promoter elements) can be recognized by specific proteins
(trans-activating factors) that ultimately regulate the stability of mRNA, and therefore, its half-life (Lopez Farre and Casado, 2001). The stability of mRNA has important implication in diseases such as heart failure, where the 3’-untranslated region has been established to control mRNA stability for important cardiovascular targets such as β-adrenergic and angiotensin type II receptors (Blaxall et al., 2000; Pende et al., 1996; Port et al., 1992) among others. In these mRNA, the 3’ untranslated region contains clusters of AU pentamers (AUUUA) and nonamers (UAAUUUAUAU) as well as GU pentamers (GUUUG) which are the specific cis-acting elements necessary for mRNA destabilization (Aharon and Schneider, 1993; Bonnieu et al., 1988; Chen and Shyu, 1994).

As mentioned above, the 3’ untranslated region of β1-adrenergic receptor mRNA, along with the trans-activating factors AUF1 and HuR, and heterogeneous nuclear riboprotein-A1, may mediate mRNA stability in heart failure. This mechanism may be responsible for the decrease in the expression of both β1-adrenergic receptor mRNA and protein (Blaxall et al., 2000; Pende et al., 1996; Port et al., 1992). Interestingly, the 3’ untranslated region of the β1-adrenergic receptor and SERCA2a mRNA both contain the AU nonamer sequences that bind to the trans-activating factors AUF1 (destabilizing) and HuR (stabilizing) (Misquitta et al., 2001, 2002). Furthermore, oxidative stress may play a role in regulating the trans-activating factors important for SERCA2 stability (Grover and Samson, 1997; Misquitta et al., 2001, 2002). Taken together, these findings may implicate a pathophysio logic role for ROS in the post-transcriptional down regulation of
SERCA2a observed in heart failure. It is unknown, however, if the decreases in SERCA2 protein and mRNA levels observed by our group and others (Arai et al., 1994; Gwathmey et al., 1987; Kennedy et al., 2003) are due to alterations in mRNA stability.

**Reactive Oxygen Species and SERCA2a Transcription**

Alternatively, ROS could act transcriptionally to decrease SERCA2a expression by acting on the SERCA2a gene. This idea seems to be supported by various models which have linked ROS indirectly to transcriptional control of SERCA2 (Aoyagi et al., 1999; Hartong et al., 1994; Rohrer et al., 1991; Thuerauf et al., 2001). Indeed there appears to be a ROS susceptible element between nucleotides –284 and –72 of the 5’ upstream region relative to the SERCA2a transcription initiation site (Arai et al., 2000).

Alternatively, decompensated (severe pressure overload) but not compensated (DOCA-salt model) hypertrophy, the region extending to –1810 appeared to be sufficient for the down regulation of SERCA2a expression (Aoyagi et al., 1999). Of further importance, Thuerauf and colleagues presented the first study in cardiomyocytes demonstrating that ATF6 binds the endoplasmic reticulum stress element 1 (position -78) and induces SERCA2 transcription following depletion of sarcoplasmic reticulum Ca\(^{2+}\) load (Thuerauf et al., 2001). A thyroid responsive element appears to be present between nucleotides –322 and –262 (50,106). More recently, site directed mutagenesis studies revealed two Sp1 sites in the SERCA2 gene promoter region that mediate the response to carvedilol under oxidative stress (Koitabashi et al., 2005).
Reactive Oxygen Species and Modulation of the SERCA2a Protein

As ROS convert some amino acid residues into derivatives of aldehydes and ketones, the accumulation of carbonyl groups has proven to be a useful measure of oxidative protein modification associated with various conditions of oxidative stress (reviewed in (Ghezzi and Bonetto, 2003; Stadtman and Berlett, 1997). In addition, changes in SERCA function may be due to lipid peroxidation as oxygen radicals have been shown to promote lipid peroxidation in sarcoplasmic reticulum membranes (Kramer et al., 1984).

Peroxynitrite, formed by the combination of super oxide anion and nitric oxide, mediates potent and preferential oxidation of thiols such as cysteine (Beckman et al., 1990). Ouabain treatment lends itself to peroxynitrite formation as ouabain has been demonstrated to increase both levels of nitric oxide (Dong et al., 2004; Xie et al., 1993) and ROS (Xie, 2003; Xie et al., 1999). Oxygen radicals also have been shown to modify SERCA activity directly at the level of protein modification (Barnes et al., 2000; Grover and Samson, 1997; Grover et al., 1997; Yamada and Ikemoto, 1978). Thiol containing peptides present particularly sensitive targets for oxidative modifications (Amici et al., 1989; Stadtman, 1993). As SERCA2a contains 29 cysteine residues, it is very possible that ROS mediated changes in SERCA function may be due to the effect on free sulphydryl groups. Interestingly, a number of groups have found that a peroxinitrite mediated process is capable of oxidizing free cysteines to disulfides in the SERCA1
isoform (Viner et al., 1996) and that modification of the Cys$^{344}$ and Cys$^{349}$ residues are critical in this process (Viner et al., 1999). Furthermore, Viner and coworkers noted that modification of Cys$^{349}$ is sufficient to significantly decrease SERCA1 activity in skeletal muscle (Viner et al., 2000) and that the SERCA2a isoform was up to four times more susceptible to oxidative modification by peroxynitrite than SERCA1 (Viner et al., 1999). Finally, Adachi and coworkers found that peroxynitrite can increase SERCA activity by S-glutathiolation at the key reactive Cys$^{674}$ residue and that this process is impaired in the setting of atherosclerosis by irreversible oxidation of the key reactive thiol(s) on SERCA (Adachi et al., 2004).
Renal Insufficiency As A Predictor of Adverse Events and Mortality

After Renal Artery Stent Placement

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ABSTRACT

Background: Renal artery stenosis is associated with substantial morbidity and mortality. This relationship is strongest in the presence of renal insufficiency (RI). The goal of this study was to assess the relationship between RI, mortality, and adverse events in the setting of renovascular disease.

Methods: Two hundred sixty-one (261) patients with significant renal artery stenosis treated with endovascular stenting were followed prospectively for 21±18 (range 0-85) months. Blood pressure (BP), renal function, medication use, and comorbidities were assessed. Death was verified through the Social Security Death Master Index File and cause of death was derived from death certificates. Medical records of 230 subjects were subsequently reviewed to identify adverse cardiovascular and renal events.

Results: Overall, 37% of patients experienced at least one adverse event post-procedure. Myocardial infarction (MI) and congestive heart failure (CHF) events increased with the degree of baseline RI.

Seventy-three deaths (28%) occurred post-procedure (range 13-2457 days). Baseline characteristics associated with mortality included: advanced age, decreased use of beta blockers, increased use of diuretics, increased serum creatinine (Cr), decreased creatinine clearance (CrCl), bilateral stenoses or stenosis of a solitary kidney, history of CHF, history of MI. Follow-up characteristics associated with mortality included: lower systolic and diastolic BP, increased serum Cr, and decreased CrCl. RI at baseline and follow-up remained associated with mortality after adjusting for other clinically and statistically significant variables. Patients whose renal function improved after stenting
appeared to demonstrate improved survival over those who did not (45% vs. 0% cumulative survival, p<0.05).

**Conclusions:** In patients with renal artery stenosis undergoing stent therapy, baseline renal insufficiency is associated with an increased incidence of adverse events as well as decreased survival, independent of other baseline clinical factors. Importantly, improvement in renal function appears to be associated with increased survival.

**Index words:** renal artery stenosis, renal insufficiency, adverse events, mortality
Treatment of renal artery stenosis (RAS) is controversial, although use of endovascular stents has been advocated (1). Several studies suggest that percutaneous revascularization may stabilize or slow progression of renal insufficiency (RI) (2-4). However, the clinical implications of stabilizing renal function are not known. Specifically, the effect of mild or moderate RI on clinical events remains unclear. Thus, some suggest a limited role for renal revascularization, reserving treatment for severe RI or for hypertension refractory to medical therapy. Others suggest a more liberal role to achieve blood pressure (BP) control and to preserve renal function.

In addition to causing hypertension and RI, RAS is associated with a high rate of fatal and non-fatal cardiovascular, renal and neurologic events (5-7). The presence of RI may be a predictor of outcomes in this group (8-11). Whether the association of RI with morbidity and mortality is causal remains to be determined. Renal insufficiency may, in this population, serve only as a marker for other conditions that alter survival including hypertension, diabetes, coronary artery disease, or heart failure (12,13). Alternatively, RI may have a causal role for the occurrence of adverse cardiovascular events. Several lines of evidence support the notion that RI may activate mechanisms implicated in atherosclerosis progression and development of left ventricular hypertrophy (14-18), both of which are prevalent in renal artery stenosis (19) and relevant to survival.

As it appears the last study to chronicle event rates in patients with RAS was performed over 30 years ago (6), we sought to document adverse events rates and survival after
renal artery revascularization and to understand the role of renal failure in their occurrence. The current study therefore addressed 2 related hypotheses. First, we sought to determine whether renal function was an independent predictor of fatal and non-fatal adverse events, after controlling for other important clinical factors. Second, we sought to determine whether improvement in renal function, after revascularization, is associated with improvement in survival.
Methods

Patient Selection

After obtaining approval from the institutional review board, patients presenting for renal angiography were offered participation in a prospective cohort study evaluating the safety and efficacy of renal artery stenting in the treatment of renal artery stenosis. Patients who gave informed consent were considered eligible for the study.

Population

Between July, 1993 and November, 2001, 261 patients with significant renal artery stenosis involving 359 renal arteries treated with endovascular stent implantation were enrolled. The majority of patients had atherosclerotic renal artery stenosis (253/261, 97%). The mean length of follow-up was 21±18 months (range 1-85 months). The baseline characteristics are shown in Table I.

Assessment of Renal Artery Stenosis

A significant renal artery stenosis was defined as a ≥60% diameter stenosis and/or a trans-lesional systolic pressure gradient of ≥ 20 mm Hg. Stenoses were quantified by using the digital caliper technique (20). Procedural success was defined as a residual stenosis of <30%.

Renal Artery Stent Procedure
Renal artery stent placement was performed using standard techniques (21). In most cases, stent implantation was performed immediately following angiography. As part of routine clinical practice at our institution, all patients received intravenous hydration prior to the procedure if RI was present. Medications administered immediately prior to stent placement included heparin and aspirin. Aspirin, 325 mg per day, was administered indefinitely thereafter. Accessory renal arteries were treated when significant stenoses, as defined above, were present. Clinically indicated repeat renal angiography was performed in 64/261 (25%) patients.

Data Collected

As part of routine clinical practice, all patients had a complete cardiovascular history and physical prior to the angiographic procedure. Coronary artery disease was identified angiographically or by history. Follow-up included outpatient clinic visits at 1, 3, 6, 12 months and yearly thereafter. At each visit current medications, BP, and serum creatinine (Cr) were recorded. The selection of antihypertensive medications was at the discretion of the treating physician.

Analysis of Blood Pressure and Renal Function

Blood pressure and renal function were assessed at baseline and each follow-up visit. At least 1 follow-up BP value and medication list were recorded for all patients surviving to the first follow-up visit. Systemic hypertension was defined as BP ≥140/90 mm Hg (22).
When multiple BP readings were available during the month before stenting, an average of at least 2 seated BP measurements was used to determine baseline blood pressure.

Baseline renal function was determined by Cr values obtained no more than one month prior to the initial renal angioplasty and stenting procedure. When multiple Cr values were available during the month before stenting, an average of at least 2 Cr values was used. Baseline Cr values were obtained in 260 (99%) patients, while follow-up Cr values were obtained in 257 patients (98%). Of patients with baseline Cr ≥ 1.6 mg/dL (≥ 141 umol/L), 73/74 (99%) had ≥1 follow-up determination. Creatinine clearance was estimated using the Cockcroft and Gault formula (23) and normalized to body surface area (24). A clinically significant change in CrCl was defined as an increase or decrease of at least 10%. Renal insufficiency was defined as CrCl ≤ 40 mL/min (≤ 0.67 mL/s).

**Survival and Adverse Event Analyses**

As part of a planned survival analysis, deaths were accounted for in the entire cohort (n=261) through both clinical follow-up and searching the Social Security Death Master Index File, a national registry indexing >95% of U.S. citizen deaths. Death certificates were obtained for 64 (88%) of the 73 deceased patients. Cause of death was determined by a committee of 3 physicians unaware of patients’ renal function.

In order to further investigate the role of renal insufficiency in morbidity and mortality, we performed a secondary, retrospective analysis of adverse events. The medical records
of 230 (88%) patients whose follow-up was completed at our institution were reviewed by a single nurse to identify fatal and non-fatal cardiovascular and renal events. Thirty-one (12%) patients in whom clinical follow-up was not completed at our institution were omitted from the adverse event analysis as the frequency of events could not be accurately assessed.

Cardiovascular events included myocardial infarction (MI), congestive heart failure (CHF), occurrence of uncontrolled hypertension (HTN), stroke, and cardiovascular death. Fatal and non-fatal cardiovascular and renal events were defined as follows:

1) MI: A patient was considered to have a myocardial infarction if two of the following criteria were met: i) elevated cardiac enzymes, ii) electrocardiographic changes or iii) a history of typical chest pain. Enzymes were considered positive for MI if the total creatine kinase (CK) was < 200 U/l and the Creatine Kinase MB isoenzyme (CKMB) was greater than 10 mg/ml or if the total CK was > 200 U/l and the CKMB index was greater than 1.9. Electrocardiographic changes consistent with MI included one or more millimeters of ST segment elevation in 2 contiguous leads or the development of new pathologic Q waves.

2) CHF: A patient was considered to have a significant episode of CHF if it resulted in hospital admission. An admission was attributed to CHF based upon patient complaints of increased dyspnea and/or edema. This diagnosis was then corroborated by the presence of objective findings documented in the patient’s
medical record. These objective findings include increased jugular venous pressure, rales, a third heart sound, edema, a chest X-ray with evidence of pulmonary edema, or elevated filling pressures documented by right heart catheterization.

3) Stroke: Defined as a new lesion consistent with a stroke documented on a brain CT or MRI as read by a radiologist.

4) Uncontrolled HTN: Defined as systolic BP $\geq$ 200 mm Hg or a diastolic BP $\geq$ 100 mm Hg on two consecutive visits in a medically compliant patient.

5) Renal events: Defined as initiation of renal replacement therapy or a 30% increase in Cr from baseline persistent after adjustment of anti-hypertensive medications and treatment of volume depletion or other reversible causes of renal dysfunction (25).

Statistical Analysis

Univariate analyses were performed with chi-square analysis and paired and unpaired t-tests as appropriate. The data are presented as mean $\pm$ one standard deviation. Statistical significance is defined as $p < 0.05$. Statistical analysis was performed using commercially available software (StatView, version 5.0, Abacus Concepts, Inc.; SAS, version 6.12, SAS Institute, Inc.)

The Mantel-Haenszel $\chi^2$ test was performed to assess the relationship between renal function and adverse events and mortality. Survival curves were constructed using the
Kaplan-Meier method and logrank tests were performed to assess the effect of renal function on adverse events and survival. Creatinine clearance was defined as either normal to mildly impaired, >40 mL/min, (> 0.67 mL/s), or moderate to severely impaired, ≤40 mL/min (≤ 0.67 mL/s), at baseline and follow-up.

Stepwise Cox Proportional Hazards models were built to adjust for the clinically significant characteristics (see Tables II and III) which, in addition to RI at baseline or follow-up, might influence adverse events and mortality. Statistically significant characteristics which were entered into the model for adverse events included: diabetes, bilateral stenoses or stenosis in solitary kidney, baseline history of CHF, number of vessels revascularized, and number of antihypertensive medications. Statistically significant characteristics which were entered into the model for mortality included: age, use of beta-blockers or diuretics at baseline, bilateral stenoses or stenosis in solitary kidney, baseline history of CHF, baseline history of MI, and follow-up systolic or diastolic BP. Criteria for entry into the models was 0.15 and criteria to stay in the models was 0.1.
Results

Procedural Outcomes

Procedural success was achieved in 260/261 patients (99%) in whom stent placement was attempted. A total of 359 vessels were treated with angioplasty and stent placement; 173 patients had 1 vessel stented, 81 had 2 vessels stented, and 7 had 3 or more vessels stented. Stenoses were reduced from 70±12% to -1±11% (p <0.0001) for the group, with the -1% residual stenosis after the procedure indicating that the final diameter of the stented region was slightly larger than the adjacent reference vessel diameter. In 1 patient, symptoms of atheroemboli were noted 1 month post procedure. On follow-up angiography performed 17±18 mo after stenting in 64 patients, restenosis was found in 31 (48% of those restudied, 12% of the entire cohort). Two patients developed total occlusion of the stented artery after stent placement. Additional stents were placed in 6 previously untreated renal arteries. Repeat revascularization was successful in 35/37 (96%) of patients.

Effects on Blood Pressure

Systolic BP (168±27 vs. 149±26 mm Hg, p <0.0001) and diastolic BP (82±15 vs. 76±14 mm Hg, p<0.0001) were lower at last follow-up (21±18 months). The number of antihypertensive medications was unchanged at last follow-up (2.3±1.0 to 2.4±1.2, p=NS).
**Effects on Renal Function**

There was no change in the serum Cr concentration at last follow-up (1.5±1.1 vs. 1.5±0.9 mg/dl, or 133±97 vs. 133±80 umol/L, p=NS), however, normalized CrCl decreased slightly (51±23 vs. 49±23 mL/min, or 0.85±0.38 vs. 0.82±0.38 mL/s, p<0.05). Among the 169 patients with baseline CrCl >40 mL/min (> 0.67 mL/s), who also had follow-up determinations, 70 (41%) had a clinically significant worsening of CrCl at last follow-up. In the 87 patients with CrCl ≤40 mL/min (≤ 0.67 mL/s), who also had follow-up determinations, 32 (37%) showed clinically significant improvement at last follow-up (23±9 to 32±12 mL/min, or 0.38±0.15 vs. 0.53±0.20 mL/s, p<0.001), 26 (30%) showed no change (32±6 vs. 32±6 mL/min, or 0.53±0.10 vs. 0.53±0.10 mL/s, p=NS), and 29 (33%) worsened (27±7 vs. 20±7 mL/min, or 0.45±0.12 vs. 0.33±0.12, p<0.001).

**Adverse Events**

During the follow-up period, the incidence of adverse events was as follows: MI 24/230 (11%), CHF 46/230 (20%), renal event 32/230 (13%), stroke 15/230 (7%), HTN event 9/230 (4%). Overall 85/230 (39%) of patients experienced at least one adverse event. Baseline and follow-up characteristics associated with adverse events are presented in Tables II and III.

These event rates were then stratified by the estimated baseline CrCl. Patients were placed into one of two groups: normal to mildly impaired renal function, CrCl ≥ 40
mL/min ($\geq 0.67$ mL/s), and moderate to severely impaired renal function, CrCl < 40 mL/min (<0.67 mL/s). A chi-square analysis was then performed for events. Compared to patients with a normal to mildly impaired CrCl, patients with moderate to severely impaired baseline clearance were at greater risk of: MI (18% vs 7%, p<0.01), CHF (34% vs 13%, p<0.001), and any event (49% vs 30%, p<0.01). Those patients with moderate to severely impaired baseline clearance also had higher incidence of any renal event (18% vs 11%) and initiation of dialysis (5% vs 1%), although these differences were not statistically significant. There was also no statistical difference in stroke rates, (6% vs 7%, p=NS). Interestingly, patients with abnormal baseline CrCl seemed less likely to experience an uncontrolled hypertensive event than patients with a normal baseline clearance (0% vs 6%, p<0.05).

Mantel-Haenszel $\chi^2$ analysis demonstrated a significant, linear trend toward increased incidence of any adverse event with decreased renal function (Figure 1). Importantly, there was no significant difference in the prevalence of coronary artery disease among the groups stratified by renal function: 21% for $\leq 20$ mL/min ($\leq 0.34$ mL/s); 13% for 21-40 mL/min (0.35-0.67 mL/s); 17% for 41-60 mL/min (0.68-1.0 mL/s); 28% for $> 60$ mL/min ($> 1.0$ mL/s); p=0.11).

Patients whose renal function worsened from normal or mildly impaired to moderately or severely impaired had significantly worse cumulative event free survival than those whose renal function remained stable (19% vs. 31%, p<0.05, Figure 2). Conversely,
patients whose renal function improved from moderately or severely impaired to normal
or mildly impaired had better event free survival than those who did not improve,
although this difference was not statistically significant (50% vs 23%, p=0.06). Finally,
in a multivariate model (Cox Proportional Hazards), RI was associated with adverse
events (RR=2.5, p=0.001) independent of CHF (RR=1.5, p=0.06) and other possible
confounding factors (Table IV).

Survival Analysis
There were 73 deaths (28% of the entire cohort) during the follow-up period. Death
occurred a mean of 21±19 months post-procedure (range 13-2457 days). Baseline and
follow-up characteristics associated with survival are presented in Tables II and III.

The cause of death was determined in 64 of the 73 deceased patients (88%).
Cardiovascular and renal causes accounted for 44/64 deaths (69%). To determine if the
degree of RI had an effect on mortality, all cause and cardiovascular and renal mortality
were plotted by CrCl. Mantel-Haenszel χ² analysis demonstrated a significant, linear
trend toward increased all cause and cardiovascular/renal mortality with decreased renal
function (Mantel-Haenszel p=0.001 and p=0.001 respectively, Figure 1).

Patients whose renal function improved from moderately or severely impaired to mildly
impaired or normal had significantly better cumulative survival than those whose renal
function did not improve (45% vs. 0%, p<0.05, Figure 3). Finally, in a multivariate
model (Cox Proportional Hazards), RI was associated with cardiovascular/ renal mortality (RR=1.9, p=0.01) independent of CHF (RR=3.2, p=0.0001) and other possible confounding factors (Table V).
Discussion

It is now well known that reductions in GFR in the pre-end stage renal disease population are a predictor of adverse events and mortality in many forms of renal disease (26-30). With renal artery atherosclerosis these relationships are not as established. There is limited data that suggest that the severity of atherosclerotic involvement (31) and RI are factors that are associated with increased mortality after renal revascularization (8-11,32). However, it is unclear whether these relationships are independent of other important clinical variables, such as the presence of diabetes or coronary artery disease. Furthermore, the linkage between RI and late adverse events in this population is not well understood.

Renal artery stenosis has been shown to be associated with coronary artery disease (33) and coronary artery disease may be an independent predictor of RAS progression (34). Furthermore, at autopsy, 12% of patients with evidence of MI were found to have significant RAS (35) and nearly 11% of patients who died from stroke were found to have RAS (36). The association with stroke is not surprising as a number of studies have shown that carotid artery atherosclerosis is more common and more severe when RAS is present (37-40). Renal artery stenosis also has been linked to end stage renal disease and the need for dialysis. In fact, RAS may contribute to up to 16% of the cases of end stage renal disease (41-43). Once RAS has been identified, as many as 30% of the patients may develop end stage renal disease (44). Furthermore end stage renal disease due to RAS
may be associated with worse outcomes than end stage renal disease due to other causes (29,42).

The current study suggests that RI is a significant predictor of both adverse events and cardiovascular mortality in this population, consistent with other published series (8-11). The relationship between RI and adverse events is strengthened by the significant decline in event free survival with advancing RI observed in this series. Similarly, the relationship between RI and cardiovascular and renal mortality is strengthened by the significant decline in survival with advancing RI. Furthermore, the prevalence of coronary artery disease was equivalent across the four categories of renal function by which we classified our patients suggesting that advanced atherosclerosis is not the primary causative factor of mortality and events in this group. These findings are consistent with observations from trials of patients with presumed essential hypertension as an etiology for RI, where chronic or even mild renal insufficiency have been implicated as independent risk factors associated with increased cardiovascular events (30,45,46). Importantly, this relationship between RI, mortality, and event free survival was not attributable to the presence of coronary artery disease, diabetes or other confounding factors as evidenced by our multivariate models.

While our finding that RI is an important predictor of survival confirms the trend in the literature, the current study serves to extend this by examining those patients who presented with abnormal renal functioning which normalized after revascularization and
vice versa. While declining renal function was associated with lower event free survival (Figure 2), there is an encouraging trend that improvement in renal function was associated with better survival (Figure 3). Moreover, there is a significant linear trend of increased adverse events as well as all cause and cardiovascular mortality in patients with decreased baseline CrCl (Figure 1).

To be sure, the majority of this study group had relatively preserved renal function with only 74/261 (28%) patients having baseline \( \text{Cr} \geq 1.6 \text{ mg/dL} \) (\( \geq 141 \text{ mL/s} \)) and 88/261 (34%) having an estimated creatinine clearance \( \leq 40 \text{ mL/min} \) (\( \leq 0.67 \text{ mL/s} \)). While these numbers limit interpretation of results to a degree, this series provides a unique opportunity to examine patients with a wide spectrum of renal function from normal to severe dysfunction. Nevertheless, these findings will need evaluation in prospective trials with sufficient power to clearly identify whether renal artery stenting, when compared to medical therapy, improves long term renal function, and whether this leads to the improvement in survival.

**Conclusion**

In patients with renal artery stenosis undergoing stent therapy, baseline renal insufficiency is associated with higher rates of fatal and non-fatal cardiovascular and renal adverse events. In contrast, improvement in renal function may be associated with increased survival.
Acknowledgements

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References


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43. Scoble J, Sweny P, Stansby G, Hamilton G: Patients with atherosclerotic renovascular


Table I. Baseline Characteristics of Patients.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study patient population, n</td>
<td>261</td>
</tr>
<tr>
<td>Age, y</td>
<td>70±10</td>
</tr>
<tr>
<td>Sex, (M/F), n/n</td>
<td>107/154</td>
</tr>
<tr>
<td>Race</td>
<td></td>
</tr>
<tr>
<td>White, non Hispanic, n (%)</td>
<td>241 (92%)</td>
</tr>
<tr>
<td>Black, n (%)</td>
<td>10 (4%)</td>
</tr>
<tr>
<td>Hispanic, n (%)</td>
<td>10 (4%)</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>75.2±16.1</td>
</tr>
<tr>
<td>Diabetes Mellitus, n (%)</td>
<td>81 (31%)</td>
</tr>
<tr>
<td>Renal Insufficiency, n (%)</td>
<td>88 (34%)</td>
</tr>
<tr>
<td>Bilateral stenoses or solitary kidney stenosis, n (%)</td>
<td>99 (38%)</td>
</tr>
<tr>
<td>Dialysis dependent, n (%)</td>
<td>5 (2%)</td>
</tr>
<tr>
<td>Coronary artery bypass graft, n (%)</td>
<td>88 (34%)</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>123 (47%)</td>
</tr>
<tr>
<td>Congestive heart failure, n (%)</td>
<td>84 (32%)</td>
</tr>
<tr>
<td>Myocardial infarction, n (%)</td>
<td>90 (34%)</td>
</tr>
<tr>
<td>Coronary artery disease, n (%)</td>
<td>210 (80%)</td>
</tr>
<tr>
<td>Cerebrovascular disease, n (%)</td>
<td>62 (24%)</td>
</tr>
<tr>
<td>Aortic aneurysm, n (%)</td>
<td>25 (10%)</td>
</tr>
</tbody>
</table>
### Indication* for Renal Angiogram

<table>
<thead>
<tr>
<th>Indication</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension, n (%)</td>
<td>237 (91%)</td>
</tr>
<tr>
<td>Congestive heart failure, n (%)</td>
<td>60 (23%)</td>
</tr>
<tr>
<td>Renal insufficiency, n (%)</td>
<td>65 (25%)</td>
</tr>
<tr>
<td>Other†, n (%)</td>
<td>17 (7%)</td>
</tr>
</tbody>
</table>

Values are mean±SD or number and percentage of patients. * Indication refers to the primary reason for the initial angiographic referral, and categories are not mutually exclusive. † Refers to incidental finding during cardiac catheterization.
Table II. Baseline Characteristics of the Patients Based on All Adverse Events (n=230) or Mortality (n=261).

<table>
<thead>
<tr>
<th></th>
<th>Patients w/ Events (n=85)</th>
<th>Event-Free Patients (n=145)</th>
<th>Deceased Patients (n=73)</th>
<th>Alive Patients (n=188)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>70±10</td>
<td>70±10</td>
<td>NS</td>
<td>72±8</td>
<td>68±11</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>32 (38%)</td>
<td>60 (41%)</td>
<td>NS</td>
<td>35 (48%)</td>
<td>72 (38%)</td>
</tr>
<tr>
<td>White, non Hispanic, n (%)</td>
<td>76 (89%)</td>
<td>136 (94%)</td>
<td>NS</td>
<td>68 (93%)</td>
<td>173 (92%)</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>36 (42%)</td>
<td>34 (23%)</td>
<td>&lt;0.01</td>
<td>29 (40%)</td>
<td>52 (28%)</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>42 (49%)</td>
<td>67 (46%)</td>
<td>NS</td>
<td>38 (52%)</td>
<td>100 (53%)</td>
</tr>
</tbody>
</table>

**Blood Pressure – mm Hg**

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic</td>
<td>172±27</td>
<td>167±28</td>
<td>NS</td>
<td>167±32</td>
<td>169±25</td>
</tr>
<tr>
<td>Diastolic</td>
<td>83±17</td>
<td>82±14</td>
<td>NS</td>
<td>83±19</td>
<td>81±13</td>
</tr>
<tr>
<td>Pulse pressure</td>
<td>89±21</td>
<td>85±24</td>
<td>NS</td>
<td>85±23</td>
<td>87±22</td>
</tr>
<tr>
<td>Antihypertensive drugs</td>
<td>2.4±1.0</td>
<td>2.2±1.0</td>
<td>NS</td>
<td>2.3±0.9</td>
<td>2.3±1.0</td>
</tr>
<tr>
<td>ACE inhibitors, n (%)</td>
<td>49 (58%)</td>
<td>71 (49%)</td>
<td>NS</td>
<td>39 (53%)</td>
<td>97 (52%)</td>
</tr>
<tr>
<td>Condition</td>
<td>Control, n (%)</td>
<td>Test, n (%)</td>
<td>p-value</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>----------------</td>
<td>-------------</td>
<td>---------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta blockers</td>
<td>43 (51%)</td>
<td>75 (52%)</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diuretics</td>
<td>42 (49%)</td>
<td>70 (48%)</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Renal Function**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control, Mean±SD</th>
<th>Test, Mean±SD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum creatinine – mg/dl</td>
<td>1.6±0.9</td>
<td>1.4±1.0</td>
<td>NS</td>
</tr>
<tr>
<td>Normalized creatinine clearance – mL/min</td>
<td>45±20</td>
<td>55±24</td>
<td>0.001</td>
</tr>
<tr>
<td>BUN – mg/dl</td>
<td>28±18</td>
<td>23±19</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Cardiac Disease**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control, n (%)</th>
<th>Test, n (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary artery disease</td>
<td>72 (85%)</td>
<td>113 (78%)</td>
<td>NS</td>
</tr>
<tr>
<td>Congestive heart failure</td>
<td>38 (45%)</td>
<td>34 (23%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>33 (39%)</td>
<td>42 (29%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

<0.01
**Indication** for Renal Angiogram

<table>
<thead>
<tr>
<th>Indication</th>
<th>Hypertension, n (%)</th>
<th>Congestive heart failure, n (%)</th>
<th>Renal insufficiency, n (%)</th>
<th>Other†, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension, n (%)</td>
<td>75 (88%)</td>
<td>27 (32%)</td>
<td>137 (94%)</td>
<td>6 (7%)</td>
</tr>
<tr>
<td>Congestive heart failure, n (%)</td>
<td>25 (17%)</td>
<td>27 (32%)</td>
<td>19 (17%)</td>
<td>10 (7%)</td>
</tr>
<tr>
<td>Renal insufficiency, n (%)</td>
<td>0.01</td>
<td>&lt;0.05</td>
<td>34 (47%)</td>
<td>2 (3%)</td>
</tr>
<tr>
<td>Other†, n (%)</td>
<td>NS</td>
<td>NS</td>
<td>31 (16%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Stenosis**

<table>
<thead>
<tr>
<th>Stenosis</th>
<th>Pre-procedure, %</th>
<th>No. vessels revascularized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-procedure, %</td>
<td>70±11</td>
<td>1.5±0.6</td>
</tr>
<tr>
<td>No. vessels revascularized</td>
<td>69±12</td>
<td>1.3±0.6</td>
</tr>
</tbody>
</table>

Values are mean±SD or number and percentage of patients. ACE = angiotensin converting enzyme. BUN = blood urea nitrogen. * Indication refers to the primary reason for the initial angiographic referral, and categories are not mutually exclusive. † Refers to incidental finding during cardiac cathererization. To convert serum creatinine in mg/dL to μmol/L,
multiply by 88.4; creatinine clearance in mL/min to mL/s, multiply by 0.01667; urea nitrogen in mg/dL to mmol/L, multiply by 0.357.
<table>
<thead>
<tr>
<th></th>
<th>Patients w/ Events (n=85)</th>
<th>Event-Free Patients (n=145)</th>
<th>Deceased Patients (n=73)</th>
<th>Alive Patients (n=188)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of time to last follow-up – months</td>
<td>24±21</td>
<td>21±17</td>
<td>21±19</td>
<td>21±18</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Blood Pressure – mm Hg</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>149±29</td>
<td>150±26</td>
<td>142±29</td>
<td>151±25</td>
<td>0.01</td>
</tr>
<tr>
<td>Diastolic</td>
<td>75±13</td>
<td>77±15</td>
<td>73±15</td>
<td>77±13</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Pulse pressure</td>
<td>74±25</td>
<td>72±20</td>
<td>69±23</td>
<td>74±21</td>
<td>NS</td>
</tr>
<tr>
<td>Antihypertensive drugs</td>
<td>2.6±1.1</td>
<td>2.3±1.2</td>
<td>2.5±1.2</td>
<td>2.4±1.2</td>
<td>NS</td>
</tr>
<tr>
<td>Total no. medications</td>
<td>8.0±3.0</td>
<td>7.4±3.0</td>
<td>7.8±3.5</td>
<td>7.4±2.9</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Renal Function</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum Creatinine – mg/dl</td>
<td>1.8±1.0</td>
<td>1.3±0.7</td>
<td>2.0±1.3</td>
<td>1.3±0.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
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<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Normalized creatinine clearance – mL/min</td>
<td>40±20</td>
<td>55±24</td>
<td>&lt;0.0001</td>
<td>39±23</td>
<td>53±22</td>
</tr>
<tr>
<td>BUN – mg/dl</td>
<td>31±17</td>
<td>25±15</td>
<td>&lt;0.01</td>
<td>34±21</td>
<td>24±12</td>
</tr>
</tbody>
</table>

Values are mean±SD or number and percentage of patients. BUN = blood urea nitrogen. To convert serum creatinine in mg/dL to µmol/L, multiply by 88.4; creatinine clearance in mL/min to mL/s, multiply by 0.01667; urea nitrogen in mg/dL to mmol/L, multiply by 0.357.
### Table IV. Cox Proportional Hazard Model for Any Adverse Event.

<table>
<thead>
<tr>
<th></th>
<th>Relative Risk</th>
<th>Confidence Interval</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal insufficiency at baseline and follow-up</td>
<td>2.1</td>
<td>[1.3 – 3.2]</td>
<td>0.001</td>
</tr>
<tr>
<td>Antihypertensive medications at Follow-up</td>
<td>1.2</td>
<td>[1.0 – 1.5]</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Congestive heart failure</td>
<td>1.5</td>
<td>[1.0 – 2.4]</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Relative risks for diabetes, bilateral stenoses or stenosis in solitary kidney, or number of vessels revascularized were not independently associated with the risk of events.
Table V. Cox Proportional Hazard Model for Cardiovascular and Renal Death.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Relative Risk</th>
<th>Confidence Interval</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congestive heart failure</td>
<td>3.2</td>
<td>[1.9 – 5.4]</td>
<td>0.0001</td>
</tr>
<tr>
<td>Renal insufficiency at baseline and follow-up</td>
<td>1.9</td>
<td>[1.1 – 3.1]</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Relative risks for age, use of beta-blockers or diuretics at baseline, bilateral stenoses or stenosis in solitary kidney, baseline history of myocardial infarction, and follow-up systolic or diastolic BP were not independently associated with the risk of mortality.
Figure Legends

**Figure 1.** Incidence of adverse events assessed according to normalized creatinine clearance at baseline. Mantel-Haenszel $\chi^2$ analysis demonstrates a significant, linear trend toward increased all cause ($\dagger$, $p=0.001$) and cardiovascular/renal ($\dagger$, $p=0.001$) mortality with decreased creatinine clearance as well as increased adverse events for any adverse event ($\dagger$, $p=0.001$), congestive heart failure ($\dagger$, $p=0.001$) and myocardial infarction ($\ast$, $p<0.05$) with decreased creatinine clearance. There was also a significant, linear trend toward decreased hypertensive events with decreased creatinine clearance ($\ast$, $p<0.05$). Importantly, there was no significant difference in the prevalence of coronary artery disease among these groups. To convert creatinine clearance in mL/min to mL/s, multiply by 0.01667.

**Figure 2.** Kaplan-Meier cumulative event-free survival plot for patients after renal artery stenting. Patients with baseline normal to mild dysfunction (creatinine clearance $>40$ mL/min) stratified by normalized creatinine clearance at last follow-up ($>40$ mL/min, 31% cumulative survival; $\leq 40$ mL/min, 20% cumulative survival, Logrank $p<0.05$). To convert creatinine clearance in mL/min to mL/s, multiply by 0.01667.

**Figure 3.** Kaplan-Meier cumulative survival from all cause mortality for patients after renal artery stenting. Patients with baseline moderate to severely impaired renal function (creatinine clearance $\leq 40$ mL/min) stratified by normalized creatinine clearance at last follow-up ($>40$ mL/min, 45% cumulative survival; $\leq 40$ mL/min, 0% cumulative survival,
Logrank $p<0.05$). To convert creatinine clearance in mL/min to mL/s, multiply by 0.01667.
Figure 1.

![Bar chart showing event rates across different categories of estimated baseline creatinine clearance.](chart.png)
Figure 2.

Cumulative Event Free Survival

Time (months)

p < 0.05

Key:  
- Baseline Cr Cl > 40 ml/min; Follow-up > 40 ml/min (n=126)
- Baseline Cr Cl > 40 ml/min; Follow-up ≤ 40 ml/min (n=20)
Effect of Chronic Renal Failure on Cardiac Contractile Function, Calcium Cycling and Gene Expression of Proteins Important for Calcium Homeostasis in the Rat

David Kennedy, M.S., Eiad Omran, M.D., Sankaridrug M. Periyasamy, Ph.D., Jalaa Nadour, M.D., Anumeet Priyadarshi, M.D., James C. Willey, M.D.
Deepak Malhotra, M.D., Ph.D., Zijian Xie, Ph.D. and Joseph I. Shapiro, M.D.

The Departments of Medicine and Pharmacology, Medical College of Ohio

Short Title: Cardiac abnormalities associated with renal failure

Key Words: Sodium, potassium, ATPase, ventricular dysfunction, myocardial calcium.

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ABSTRACT

Background: Patients with chronic renal failure frequently develop cardiac hypertrophy and diastolic dysfunction. However the mechanisms by which this occurs are still unclear.

Methods: Male Sprague Dawley rats were subjected to 5/6th nephrectomy and studied for their isolated myocyte function, calcium cycling and gene expression of proteins important in calcium homeostasis after 4 weeks. Comparable rats subjected to suprarenal aortic banding for the same duration were used for comparison.

Results: Rats subjected to 5/6th nephrectomy and aortic banding developed comparable hypertension. However, rats subjected to 5/6th nephrectomy experienced a greater degree of cardiac hypertrophy and downregulation of cardiac sodium potassium ATPase (Na⁺/K⁺-ATPase) activity than rats subjected to aortic banding. Moreover, cells isolated from the 5/6th nephrectomy rat hearts displayed impaired contractile function and altered calcium cycling compared with cells isolated from control or aortic constriction rat hearts. The 5/6th nephrectomy rat heart cells displayed a prolonged time constant for calcium recovery following stimulation which corresponded to decreases in homogenate sarcoplasmic reticulum calcium ATPase-2a (SERCA2a) activity, protein density, and mRNA for SERCA2a.

Conclusion: Chronic renal failure leads to alterations in cardiac gene expression which, in turn, produces alterations in cardiac calcium cycling and contractile function. These changes cannot be explained only by the observed increases in blood pressure.
INTRODUCTION

Patients with renal failure usually develop cardiac complications. In end stage renal disease (ESRD) patients treated with hemodialysis in the United States, mortality rates exceed 20% per year with more than 50% attributed to cardiac mortality [1]. Although the term “uremic cardiomyopathy” had previously been used to refer to a dilated cardiomyopathy complicating renal failure, more recent studies suggest that the most common form of heart disease in renal failure patients is one characterized by diastolic dysfunction and left ventricular hypertrophy [2]. Although a number of known factors have been implicated in the pathogenesis of the left ventricular hypertrophy and diastolic dysfunction, our understanding of these processes are still incomplete [3].

For many years, it has been known that the sodium pump is abnormal in chronic renal failure and that a circulating inhibitor(s) can be demonstrated in the serum of uremic patients [4-6]. We have observed that sodium pump inhibition initiates a signal cascade that can cause alterations in gene transcription and ultimately produce hypertrophy in cardiomyocytes grown in culture [7-9]. We also have observed that sodium pump inhibitors, including those circulating in the serum of uremic patients, can acutely cause altered calcium cycling and cardiomyocyte relaxation through sodium pump inhibition [10]. To further examine how cardiac growth and function are chronically modified by the uremic milieu, the following studies were performed.
METHODS

Animals: Male Sprague Dawley rats (200-250 gm) were subjected to either 5/6th nephrectomy produced by removal of the right kidney and segmental infarction of 2/3rd of the remaining kidney with silk ligatures, suprarenal aortic constriction (produced by tying a silk ligature (4-0) around a 21 gauge needle and the suprarenal abdominal aorta and then removing the needle or sham surgery). The surgical approaches have been described in detail in previous publications from our laboratory [7,11]. After surgery, the rats were allowed to recover for 4 weeks having access to ad libitum food (Rodent Laboratory Chow 5001, PMI Nutrition International, Inc., Brentwood, MO) and water. The content of this chow mix is listed on the company web page, but the nutritional essentials are as follows: protein 23.4%, fat 4.5%, crude fiber 5.8% and total digestable nutrients 76%. The calcium and phosphorus contents of the chow was 1.00% and 0.61%, respectively. At the end of 4 weeks, some animals were anesthetized and the blood pressure was determined by placing a catheter in the carotid artery prior to removal of the heart for subsequent studies. In some experiments, cardiomyocytes were isolated for subsequent study. In other experiments, hearts were removed and immediately homogenized to allow for the determination of the enzymatic activities of the Na⁺/K⁺-ATPase and the SERCA2a as well as these protein densities using Western blots [7]. In other experiments, the left ventricle was quickly excised and frozen in liquid nitrogen. This frozen tissue was then stored at −80°C until it was subsequently analyzed with quantitative PCR (StaRT-PCR) for determination of mRNA for several gene products.
Isolation and culture of cardiac myocytes: Details of the method of isolation and culture of calcium tolerant adult myocytes may be found in several recent reports from our laboratory [9;10;12]. This method of isolation produced a good yield of rod-shaped (70-80%) myocytes in each of the experimental groups presented in this paper.

Measurements of the calcium transient and contractility: The calcium transient was measured by using the calcium selective fluorescent dye indo-1 and spectrofluorimeter (Photon Technology International, Monmouth Junction, NJ). Myocyte contractility was measured simultaneously using an edge detector system (Crystal Biotech, Northboro, MA) which, along with the spectrofluorimeter, was interfaced with an inverted microscope. The simultaneous observation of both indo-1 fluorescence and edge detection was accomplished by continuous illumination of the cells during field stimulation with a red light and splitting of the emission light based on wavelength to either a video imaging system or the spectrofluorimeter. Again, details of this methodology may be found in recent reports from our laboratory [9;10;12]. Calculations of cytosolic calcium concentration ([Ca^{2+}]_i) were made using the formula

\[ [\text{Ca}^{2+}]_i = \text{Kd} \times \frac{D_{\text{free}}}{D_{\text{bound}}} \times \frac{(R - R_{\text{min}})}{(R_{\text{max}} - R)} \]

where the Kd was assumed to be 250nM, the Dfree and Dbound represent the intensity of the fluorescence at 485nm following EGTA (4mM) and Ionomycin (10^{-6}M) treatment of the cell, respectively, and Rmin and Rmax were the ratios obtained under these conditions.
The time constant, \( \tau \), for recovery of length and calcium following electrical stimulation was performed by fitting a least square regression line to the log transformation of the edge detection and fluorescence data, respectively, as described by Bers, Bassani and colleagues \[13;14\] and also reported previously by our group \[10\].

**StaRT PCR:** Analysis of gene expression for proteins important in calcium homeostasis as well as markers of cardiac hypertrophy was performed using standardized reverse transcription polymerase chain reaction (StaRT-PCR) which allows for quantitative measurement of gene expression based on the ratio of native PCR products to specific competitive templates (CT) \[15\]. Detailed description of the principles, reagents, and protocol for this methodology may be found in several recent reports \[15-18\]. Briefly, left ventricles obtained from remnant and sham treated rats were homogenized and total RNA extraction was performed on the tissue homogenate as described by the TRI-REAGENT protocol (Molecular Research Center, Inc, Cincinnati, OH). Reverse transcription (RT) (5 min denaturing at 94\(^\circ\)C, 1 hour incubation at 37\(^\circ\)C, 5 min heat stopped at 94\(^\circ\)C) and PCR was then performed.

Primers for all target and housekeeping genes that were evaluated in this study are listed in Table 1. Reaction volumes were 10 ul and each contained 0.05 ug of each primer, 0.5 U Taq polymerase, 1 ul PCR buffer, 0.2 mM dNTPs, 1 ul of a CT mixture containing the desired molarity of each CT, and 1 ul cDNA diluted such that native GAPDH competed equally with the GAPDH CT present in the chosen CT mixture. The PCR reaction
mixtures were placed in capillary tubes and cycled 35 times in a Rapidcycler air
thermocycler (Idaho Technology). Each cycle consisted of 5 s at 94\(^\circ\)C, 10 s at 58\(^\circ\)C, and
15 s at 72\(^\circ\)C with a slope of 9.9, for a total amplification time of approximately 25 min.
The PCR products were electrophoresed on either DNA 7500 or 1000 assay LabChips®
(Agilent Technologies, Palo Alto, CA), and quantitative analysis was performed as
described previously [15]. Levels of expression are reported as units of messenger RNA
(mRNA)/10\(^6\) copies of GAPDH.

**Western Blot Analysis SERCA2a and Sodium Calcium Exchanger (NCX-1):** The hearts
from sham and nephrectomized rats were excised and the left ventricles were dissected
out. Left ventricles were homogenized in 25mM imidazole buffer pH 7.0 containing
protease inhibitors. An aliquot of the homogenate was removed and its protein content
was determined [19]. After solubilizing the homogenates in sample buffer (2% SDS, 5%
\(\beta\)-mercaptoethanol, 20% glycerol, 0.005% bromophenol blue and 50mM Tris-HCl pH
7.0), the proteins in the homogenates were resolved as described by Lammeli [20] on a
SDS-PAGE using 10% gel. The proteins were transferred to nitrocellulose membrane
following the method of Towbin [21] and immunoblotted with anti-SERCA2 mAb or
anti-NCX-1 mAb (Affinity Bioreagents, Inc., Golden, CO). The immunoreactive
products were visualized with horseradish peroxide conjugated to donkey anti-mouse IgG
(Affinity Bioreagents, Inc., Golden, CO) using an enhanced chemiluminescence substrate
(Pierce, Rockford, IL). The images of the immunoreactive products were quantified with
a Molecular Analyst™ software program (BioRad Laboratories, Hercules, CA).
Measurement of Na\(^+\)/K\(^+\)-ATPase Activity: Cardiac homogenates were prepared in the presence of protease inhibitors, and ouabain sensitive Na\(^+\)/K\(^+\)-ATPase activity was measured as we have previously reported [7].

Measurement of SERCA2a Activity: To measure sarcoplasmic reticulum calcium ATPase activity (which we are assuming is predominantly SERCA2a activity [22;23]), the method of Simonides and Hardeveld [24] was used with modifications. Homogenates of left ventricles of rats were prepared in a medium containing 25mM imidazole (pH 7.0) and protease inhibitors. Homogenates were then subjected to freeze-thaw cycles five times to open up the vesicles formed during the homogenization. The assay medium consists of 1.0ml containing 40 mM imidazole pH 7.0, 100mM KCl, 5mM K+ oxalate, 5mM NaN3, 3 mM MgCl2, 2mM ouabain, 200-220 µg of homogenate, 3mM(γ-32P)-ATP, and 10 µM CaCl\(_2\) or 2 mM EGTA. After a 5 min preincubation at 37\(^0\) C, enzymatic reaction was initiated by the addition of (γ-32P)-ATP and terminated 15 min later by the addition of 8% perchloric acid. Released inorganic (32P)-phosphate was measured as described by Askari et al [25]. The difference between ATPase activity in the presence and absence of CaCl\(_2\) was considered as SERCA2a activity.

Statistical Analysis: Data obtained were first tested for normality. If the data did not pass the normality test, the Tukey test (for multiple groups) or the Mann-Whitney Rank Sum test were used to compare the data. If the data did pass the normality test, parametric comparisons were performed. If more than two groups were compared, one-way analysis
of variance was performed prior to comparison of individual groups with the unpaired Student’s t-test with Bonferroni’s correction for multiple comparisons. If only two groups of normal data were compared, the Student’s t-test was used without correction [26]. Statistical analysis was performed using Sigmastat™ software. All animal experimentation described in the manuscript was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals using protocols approved by the Medical College of Ohio Institutional Animal Use and Care (IACUC) Committee.
RESULTS

Effect of 5/6th Nephrectomy on Heart Size and Blood Pressure:
The production of 5/6th nephrectomy and aortic constriction both resulted in considerable increases in both blood pressure and heart size (table 2). Although the increases in blood pressure were quite comparable to that seen in rats subjected to suprarenal aortic constriction studied at a similar time point, the degree of cardiac hypertrophy was approximately 50% greater (p< 0.05, table 2) in rats subjected to 5/6th nephrectomy. Rats subjected to aortic constriction maintained a normal hematocrit whereas chronic renal failure rats had reduced hematocrit values (table 2).

Effect of 5/6th Nephrectomy on Cardiac Na⁺/K⁺-ATPase Expression and Activity:
Rats subjected to 5/6th nephrectomy demonstrated marked decreases in Na⁺/K⁺ ATPase activity compared with sham treated rats. Although rats subjected to aortic constriction also demonstrated decreases in Na⁺/K⁺ ATPase activity, this decrease was more modest than that seen in the 5/6th nephrectomy rats (figure 1). Examining the Na⁺/K⁺ ATPase isoforms, the 5/6th nephrectomy rat hearts demonstrated a considerable decrease in the expression of both α1 and α2 isoforms whereas the decrease in Na⁺/K⁺ ATPase appeared to be confined to the α2 isoform in the aortic constriction rat hearts (figure 1).
Effect of 5/6th Nephrectomy on Isolated Cardiac Myocyte Calcium Cycling and Contractile Function:

Cardiomyocytes isolated from rats subjected to aortic constriction demonstrated no significant alterations in contractile function and calcium cycling when compared to sham operated rats after 4-6 weeks. However, cardiomyocytes isolated from 5/6th nephrectomy rats demonstrated decreases in fractional shortening as well as substantial increases in both diastolic and systolic calcium concentrations. Moreover, the rats subjected to 5/6th nephrectomy showed substantially greater $\tau$ values for both calcium and length recovery following stimulation (table 3).

Effect of 5/6th Nephrectomy on SERCA2a:

To further examine the mechanisms underlying the alterations in calcium cycling seen in the hearts of rats subjected to 5/6th nephrectomy, SERCA2a activity, protein density and gene expression were examined. Rats subjected to 5/6th nephrectomy showed substantially decreased SERCA2a activity compared with sham treated rats and rats subjected to aortic constriction (figure 2). Quantification of protein density with western blotting confirmed a decrease in SERCA2a in the 5/6th nephrectomy as compared with the sham treated rat hearts (figure 2). Interestingly, western blotting to determine the protein density of the NCX-1 revealed nearly a 100% increase in the hearts from the 5/6th nephrectomy compared with sham treated rats (1.94+0.22 vs 1.00+0.10, both N=5, $p < 0.01$).
**Effect of 5/6th Nephrectomy on Cardiac Gene Expression:**

To further examine the mechanisms underlying the biochemical and physiological changes described above, StaRT-PCR was used to quantify cardiac gene expression for the Na⁺/K⁺-ATPase isoforms, SERCA2a and NCX-1 as well as skeletal muscle actin (skACT) and atrial natriuretic peptide (ANP). Hearts isolated from 5/6th nephrectomy rats demonstrated significant decreases in message expression for the α2 isoform of Na⁺/K⁺-ATPase as well as increases in skACT and ANP (table 4). In particular, SERCA2a message was reduced by 50% (p < 0.01) in these 5/6th nephrectomy hearts.
DISCUSSION

Although the term “uremic cardiomyopathy” has been used for many years, our concept of the clinical features has changed dramatically [27]. Foley and Parfrey have demonstrated that while systolic dysfunction is demonstrable in the minority of chronic renal failure patients [28], hospitalization for fluid overload or congestive heart failure occurs very commonly [29]. Recent work suggests that echocardiographically demonstrable diastolic dysfunction is extremely common in ESRD patients treated with hemodialysis [30]. As discussed briefly in the introduction, we have observed that sodium pump inhibition, which appears to accompany the chronic renal failure state, may acutely cause or contribute to both diastolic dysfunction and cardiac hypertrophy [7;10]. To gain further insights into this issue we conducted the current study.

We observed that chronic renal failure induced by partial (5/6th) nephrectomy was associated with marked increases in cardiac size, a phenomenon which could not be accounted for only by the hypertension associated with this model. We also noted characteristic changes in message expression quite similar to that seen in ouabain induced cardiac hypertrophy in vitro. Specifically, we saw increases in the transcription of ANP and skACT which appears to accompany all pressure overload type cardiac hypertrophy [31] as well as decreases in the α2 isoform of Na⁺/K⁺ ATPase. The fall in 2 expression is analogous to the decrease in 3 expression in neonatal cardiac myocyte hypertrophy induced with ouabain [32;33] or observed with the coincident exposure to both pressure overload and potassium depletion together [7] or pressure overload alone [34]. We have
postulated that the decrease in $\alpha_2$ (or $\alpha_3$) expression may constitute a negative feedback attenuating hypertrophy induced by ouabain or other digitalis like substances [9;32;33]. In the remnant kidney cardiac cells, we noted that in addition to the decreases in $\alpha_2$ Na$^+/K^+$-ATPase, the $\alpha_1$ isoform protein expression was also significantly decreased. We did not examine $\alpha_3$ isoform expression in the current study.

We must point out that the hematocrit was diminished in the chronic renal failure rats, raising the possibility that the accelerated hypertrophy in these animals was due to an additive or synergistic effect between pressure and volume overload as suggested to occur in patients with chronic renal failure [35]. Although our gene expression data appear more consistent with a purely “pressure overload” phenotype [34], it is not possible to exclude this possibility from our data.

Our findings regarding sodium pump gene expression are submitted on a literature background which is plagued by inconsistency, at least in the case of chronic renal failure. Greiber and colleagues reported essentially no difference in $\alpha_1$ and $\alpha_2$ isoform mRNA or protein expression in a similar model of chronic renal failure studied at approximately the same time point [36]. Da Silva and colleagues found significant decreases in cardiac mRNA for $\alpha_2$ without any changes in $\alpha_2$ protein observed [37]. Bonilla and colleagues reported decreases in both Na$^+/K^+$ ATPase activity as well as mRNA for the $\alpha_1$ isoform in skeletal muscle; however, these workers found an increase
in α2 mRNA in this tissue [38]. At present, the reason(s) for the discrepancy between our findings and these other studies is (are) not clear.

Regarding the myocyte function, we found that the cells isolated from the hearts of rats bearing remnant kidney demonstrated both systolic and diastolic dysfunction in vitro. Our findings were quite similar to that reported by McMahon and colleagues in 1996 [39]. This was in contrast to the heart cells isolated from the aortic clip rats which demonstrated grossly normal function and calcium cycling at the time of isolation which was between 4-6 weeks after surgery; this observation was also consistent with previous studies performed early after induction of aortic banding [40]. The systolic dysfunction seen in the heart cells from the remnant kidney rats was strikingly similar to that observed when heart cells are isolated from rats with congestive heart failure from a variety of causes including aortic constriction [41]. In particular, we noted marked calcium insensitivity in these heart cells with the diastolic calcium value substantially elevated compared with sham treated rats. It appears that it takes considerably longer periods of aortic constriction to produce abnormal systolic function and calcium insensitivity in these isolated myocytes than the induction of uremia by the 5/6th nephrectomy model which is associated with very similar blood pressure elevation in the species and strain that we employed [42]. Although most patients with chronic renal failure display normal (or even supranormal) systolic function [2], we suggest that the digitalis like substances that circulate in such patients might mask underlying contractile problems [10].
Other investigators also have noted increases in cytosolic calcium during experimental uremia, but this has been ascribed to the associated secondary hyperparathyroidism [43]. Although we did not attempt to dissect out the role of parathyroid hormone in this current study, we have previously reported that acute sodium pump inhibition by deproteinated serum extract as well as administration of cardiac glycosides elevates cardiac cytosolic calcium [7;9;10]. We speculate that chronic exposure to such sodium pump inhibition could alter cardiac calcium cycling, contractile function and gene expression on a chronic basis. However, this speculation as well as the role that parathyroid hormone plays in the regulation of circulating inhibitors of the sodium pump requires additional study.

In addition to this systolic dysfunction, we also observed substantial diastolic dysfunction characterized by delayed recovery of length in parallel with a longer time constant $\tau$ describing calcium recovery following stimulation [44]. This also has been observed in a variety of cardiomyopathies including that induced by prolonged (> 12 weeks) aortic banding [45]. Although the decrease in cytosolic calcium following stimulation depends on several processes, the most important quantitatively are through reuptake of calcium into the sarcoplasmic reticulum and extrusion out of the cell via the sodium calcium exchanger. Bassani and colleagues reported that sarcoplasmic reticulum calcium reuptake is the major determinant of the rate of decay of the calcium transient in rat cells with the sodium calcium exchanger accounting for substantially less of this calcium recovery in the rat [46]. While the sodium calcium exchanger is responsible for the majority of calcium efflux in myocytes, it also can serve as a calcium influx mechanism [47].
Experimental and human heart failure are typically characterized by a decrease in SERCA2a [48;49] and an increase in NCX-1 [50], although it should be noted that some studies have observed NCX-1 to be unchanged or even decreased [51;52]. Thus, some authors suggest that the [Ca$^{2+}$]$_i$, is determined by the interactions between these two calcium handling proteins taken together (i.e. the ratio of NCX-1 to SERCA) rather than either one separately [53]. Thus, in order to elucidate the mechanisms responsible for deranged calcium metabolism seen with 5/6$^{th}$ nephrectomy, we focused our investigation on SERCA2a and NCX-1 as these proteins represent the predominant cardiac isoforms of both the sarcoplasmic reticulum calcium ATPase and the sodium calcium exchanger, respectively. In our setting we have decreased levels of SERCA2a and increased levels of NCX-1. It has been suggested that in pathophysiological conditions where there is a reduction in SERCA2a function, overexpression of NCX-1 may participate in a compensatory attempt to maintain normal calcium homeostasis [54]. Based on our data, it appears that transcriptional downregulation of SERCA2a leads to decreases in sarcoplasmic reticulum calcium reuptake and impaired myocyte relaxation in this model. As discussed above, it is unclear what role parathyroid hormone and circulating digitalis like substances play in the regulation of these transport proteins in the setting of chronic renal failure, and further examination of this important area is certainly warranted.

In summary, we observed that uremia induced by 5/6$^{th}$ nephrectomy caused marked cardiac hypertrophy and changes in cardiac gene expression which could not be explained by only the observed increases in blood pressure. We also noted that calcium
cycling and contractile function were deranged in the myocytes isolated from these hearts and that transcriptional downregulation of the SERCA2a may account for the impaired diastolic function seen in these myocytes.
ACKNOWLEDGEMENTS:

Some of these data were presented in abstract form at the 2000 American Society of Nephrology Meetings. The authors would like to thank Ms. Carol Woods for her excellent secretarial assistance as well as Jie Chen and Dr. Wei Han for their technical efforts. Portions of this study were supported by the American Heart Association (National and Northwest Ohio Affiliate) and the National Institutes of Health (HL57144 and HL63238 and HL67963).
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and the Na(+)/Ca(2+)Exchanger are antithetically regulated during mouse cardiac


Table 1: Primers Used For StaRT PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
<th>Position (bp)</th>
<th>Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>gAC CCC TTC ATT gAC CTC AA</td>
<td>127-147</td>
<td>NM_017008</td>
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<td></td>
<td>Reverse</td>
<td>TgC TTC ACC ACC TTC TTg AT</td>
<td>776-796</td>
<td>689</td>
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<tr>
<td></td>
<td>CT</td>
<td>R+ gAT gAC CTT gCC CAC AgC CT</td>
<td>628-668</td>
<td>581</td>
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<tr>
<td>α1Na⁺/K⁺</td>
<td>ATPase</td>
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<td></td>
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<td>552</td>
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<td></td>
<td>Reverse</td>
<td>R+ Tgg CAg TgA gCg TCA gAC ATA C</td>
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<tr>
<td>α2Na⁺/K⁺</td>
<td>ATPase</td>
<td>AgA TCA gTC Tgg ggC CAC TTT</td>
<td>1304-1325</td>
<td>NM_012505</td>
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<td></td>
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<td>1781-1802</td>
<td>519</td>
</tr>
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<td>Reverse</td>
<td>R+ ggC ATC TTg CAT CTC CTT gTC</td>
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<td>skACT</td>
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<tr>
<td></td>
<td>CT</td>
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<td></td>
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<td>SERCA2a</td>
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<td>NCX-1</td>
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<td>132</td>
</tr>
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</table>

α1Na⁺/K⁺ ATPase (Sodium Potassium Pump, α subunit, isoform 1)
α2Na⁺/K⁺ ATPase (Sodium Potassium Pump, α subunit, isoform 2)
skACT (Skeletal Actin)
ANP (Atrial Natriuretic Factor)
NCX-1 (sodium calcium exchanger)
SERCA2a (Sarcoplasmic Reticulum Calcium Atpase, isoform 2a)
GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)
Table 2: Effect of partial nephrectomy (PNx) and aortic constriction (AC) on body weight, blood pressure and heart weight.

<table>
<thead>
<tr>
<th></th>
<th>Sham (n=12)</th>
<th>AC (N=14)</th>
<th>PNx (n=14)</th>
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</thead>
<tbody>
<tr>
<td>Weight (gms)</td>
<td>467±12</td>
<td>464±9</td>
<td>458±12</td>
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<tr>
<td>Mean Arterial Pressure (mmHg)</td>
<td>109±2</td>
<td>175±8**</td>
<td>165±7**</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>51±1</td>
<td>50±2</td>
<td>38±2***##</td>
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<tr>
<td>Heart Weight (gms)</td>
<td>1.24±.03</td>
<td>1.45±.04**</td>
<td>1.56±.03**#</td>
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</tbody>
</table>

Results expressed as mean ± SEM. * p < 0.05, ** p < 0.01 vs Sham. # p < 0.05, ## p < 0.01 vs Aortic Clip.
Table 3: Effect of partial nephrectomy (PNx) and aortic constriction (AC) on contractile function and calcium cycling in isolated cardiac myocytes:

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Sham</th>
<th>AC</th>
<th>PNx</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=</td>
<td>N=</td>
<td>N=</td>
</tr>
<tr>
<td>N= 10</td>
<td>8</td>
<td>12</td>
<td></td>
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<tr>
<td><strong>Contractile Function</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fractional shortening (%)</td>
<td>13.0±</td>
<td>14.5±</td>
<td>6.8± .06*#</td>
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<tr>
<td>Time to peak (Edge) (msec)</td>
<td>161±</td>
<td>17</td>
<td>182± 21</td>
</tr>
<tr>
<td>τ (Edge) (msec)</td>
<td>155±</td>
<td>18</td>
<td>220± 14**##</td>
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<tr>
<td><strong>Calcium Metabolism</strong></td>
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<tr>
<td>Diastolic [Ca^{2+}] (nM)</td>
<td>48±</td>
<td>5</td>
<td>96± 16*#</td>
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<tr>
<td>Systolic [Ca^{2+}] (nM)</td>
<td>198±</td>
<td>44</td>
<td>336± 50*#</td>
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<tr>
<td>Time to peak (Ca^{2+}) (msec)</td>
<td>61±</td>
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<td>78± 10</td>
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<tr>
<td>τ (Ca^{2+}) (msec)</td>
<td>381±</td>
<td>17</td>
<td>494± 16***##</td>
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</table>

Data expressed as mean ± SEM. * p < 0.05 vs Sham, ** p < 0.01 vs Sham, # p < 0.05, ## p < 0.01 vs Aortic clip by unpaired t-test.
Table 4: Effect of partial nephrectomy (PNx) on cardiac gene expression:

<table>
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<tr>
<th>Gene</th>
<th>Sham (N=9)</th>
<th>PNx (N=9)</th>
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<tbody>
<tr>
<td>α1Na⁺/K⁺ ATPase</td>
<td>65.9±6.1X10³</td>
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Levels of expression are reported as units of messenger RNA (mRNA)/10⁶ copies of GAPDH. Data expressed as mean ± SEM. * p < 0.05 vs Sham, ** p < 0.01 vs Sham by unpaired t-test.
Figure Legends

Figure 1: Comparison of the Na\(^+\)/K\(^+\)-ATPase enzymatic activity as well as \(\alpha1\) and \(\alpha2\) Na/K-ATPase protein densities in homogenates of hearts isolated from sham treated (open bars, N=12), aortic constriction (light gray hatched bars, N=10) and partially nephrectomized (dark gray hatched bars, N=12) rats. * \(p < 0.05\), ** \(p < 0.01\) vs Sham, # \(p < 0.05\) vs aortic constriction.

Figure 2: Comparison of the sarcoplasmic reticulum calcium ATPase (SR-Ca\(^2+\)-ATPase) activity and protein density in homogenates of hearts isolated from sham (open bars, N=6) and partially nephrectomized (light gray hatched bars, N=6) rats. * \(p < 0.05\), ** \(p < 0.01\) vs Sham.
Figure 1.
Figure 2.
Central Role for the Cardiotonic Steroid, Marinobufagenin, in the Pathogenesis of Experimental Uremic Cardiomyopathy

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* Equal contributions to this project were made from the first and second author.

Short Title: Marinobufagenin in Uremic Cardiomyopathy

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Abstract:

Patients with chronic renal failure develop a “uremic” cardiomyopathy characterized by diastolic dysfunction, cardiac hypertrophy and systemic oxidant stress. Patients with chronic renal failure also are known to have increases in the circulating concentrations of the cardiotonic steroid marinobufagenin. On this background, we hypothesized that elevations in circulating marinobufagenin may be involved in the cardiomyopathy.

First, we observed that administration of marinobufagenin (10 μg/kg/day) for 4 weeks caused comparable increases in plasma MBG as partial nephrectomy at 4 weeks. Marinobufagenin infusion caused increases in conscious blood pressure, cardiac weight and the time constant for left ventricular relaxation similar to partial nephrectomy. Decreases in the expression of the cardiac sarcoplasmic reticulum ATPase, cardiac fibrosis and systemic oxidant stress were observed with both marinobufagenin infusion and partial nephrectomy. Next, rats were actively immunized against a marinobufagenin - bovine serum albumin conjugate or bovine serum albumin control, and partial nephrectomy was subsequently performed. Immunization against marinobufagenin attenuated the cardiac hypertrophy, impairment of diastolic function, cardiac fibrosis and systemic oxidant stress seen with partial nephrectomy without significant effect on conscious blood pressure. These data suggest that the increased concentrations of marinobufagenin are important in the cardiac disease and oxidant stress state seen with renal failure.
Key Words: Cardiomyopathy; Renal Failure, Sarcoplasmic Reticulum Calcium ATP-ase (SERCA); Cardiotonic Steroids; Reactive Oxygen Species; Fibrosis
Introduction:

The care of patients with chronic renal failure is currently complicated by their propensity to develop cardiac disease. This cardiac disease is directly responsible for much of the extremely high morbidity and mortality seen in patients with end stage renal disease (Sarnak et al., 2003). At present, the molecular basis of this “uremic cardiomyopathy” which is characterized by a systemic oxidant stress state, marked cardiac hypertrophy and diastolic dysfunction is still poorly understood. Interestingly, even mild degrees of chronic renal failure appear to confer a significant increase in cardiovascular disease (Garg et al., 2002; Henry et al., 2002).

The partial nephrectomy model in the rat has been used to simulate experimental uremia in order to study the cardiac abnormalities which accompany renal failure (Hostetter et al., 1981). A number of factors, including volume overload, have been implicated in the pathogenesis of the cardiac disease in this model (reviewed in Middleton et al., 2001). We have observed that cardiac myocytes isolated from rats subjected to partial (i.e., 5/6th) nephrectomy have diastolic dysfunction in vitro which can be attributed to reduced sarcoplasmic reticulum calcium ATPase (SERCA) activity and, in turn, appears to be dependent on proportional decreases in SERCA2a protein and mRNA (Kennedy et al., 2003). It has been observed that steroid molecules, which bind to the plasmalemmal Na/K-ATPase and have structural similarity to the medication digitalis, accumulate in renal failure. These molecules have been referred to as digitalis-like substances or, more recently, cardiotonic steroids. Considerable effort has gone into the measurement of these molecules and the elucidation of their role in cardiac and renal
physiology (de Wardener, 1996). Recent work has established that the cardiotonic steroid, marinobufagenin (MBG), induces natriuresis and, in susceptible rat strains, increases blood pressure (Fedorova et al., 2005; Fedorova et al., 2002). Elevations of circulating MBG have been clearly demonstrated in both clinical and experimental renal failure whereas another cardiotonic steroid, endogenous ouabain, does not increase at 4 weeks in experimental renal failure (Komiyama et al., 2005; Priyadarshi et al., 2003).

Interestingly, investigators postulated a role for endogenous natriuretic substances in the pathobiology of uremia decades prior to their identification (Bricker, 1972). Our group and others have observed that cardiotonic steroids induce signaling through the plasmalemmal Na/K-ATPase which resides in caveolae (Liu et al., 2005; Liu et al., 2003). This signaling requires the generation of reactive oxygen species, has genomic effects that can be attributable to modulation of transcription factors including SP-1, and induces hypertrophic changes in both neonatal and adult cardiac myocytes in vitro (Haas et al., 2000; Huang et al., 1997; Kometiani et al., 1998; Liu et al., 2000; Xie et al., 1999) (Figure 1). Recently, our group has shown that passive administration of antibodies raised against MBG reduced Na/K-ATPase endocytosis and sodium excretion in Sprague Dawley rats given a high salt diet (Periyasamy et al., 2005). On this background, we postulated that increases in circulating MBG may be in part responsible for the systemic oxidant stress state and the anatomic and functional cardiac changes seen with experimental uremia.

To test this hypothesis, we performed the following studies. As plasma MBG concentrations are elevated in rats subjected to partial nephrectomy, we administered
MBG to sham operated rats to achieve similar concentrations. Conversely, to neutralize MBG in the setting of renal failure, we actively immunized animals against MBG prior to partial nephrectomy. After performing these maneuvers, physiological, morphological and biochemical assays were performed.
Methods:

**Animals:** Male, Sprague Dawley rats were used for all studies. All animal experimentation described in the manuscript was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals using protocols approved by the Medical University of Ohio Institutional Animal Use and Care (IACUC) Committee.

**Experimental Groups:** Rats which were subjected to sham surgery and no MBG infusion or partial nephrectomy are referred to as Sham [N=18]. Rats subjected to partial nephrectomy [N=8], as well as those who received control immunization against bovine serum albumin (BSA) and partial nephrectomy [N=12], were very similar with respect to functional and biochemical analysis and were, therefore, pooled into one group and referred to as PNx [N=20]. MBG infused rats are referred to as MBG [N=20]. Rats which were immunized against MBG-BSA conjugate prior to partial nephrectomy are referred to as PNx-IM [N=18].

**MBG Infusion:** MBG was isolated from toad (Bufo Marinus) venom as described previously (Bagrov et al., 1995). The isolated MBG was > 99% pure based on HPLC and mass spectroscopy analysis. MBG was infused for a period of 4 weeks at 10 μg/kg/day with an osmotic minipump (Alzet Model 2004, Durect Corp., Cupertino, CA). The stability of MBG 4 weeks at 37°C was confirmed by comparable inhibition of $^{86}$Rb uptake in LLCPK1 cells at 2.5 X $10^{-7}$ and 1 X $10^{-6}$ M concentrations as MBG prepared immediately as well as by mass spectroscopy analysis.
Experimental renal failure: 5/6th nephrectomy (partial nephrectomy) was induced by removal of the right kidney and selective infarction of 2/3rds of the left kidney with silk ligatures as described previously (Kennedy et al., 2003).

Immunization against MBG: Rats were immunized with an MBG-BSA conjugate and subjected to 5/6th nephrectomy. The immunization schedule was three weekly injections (250 µg/kg/wk SQ) in complete Freund’s adjuvant prior to the partial nephrectomy with a last boost at the time of surgery. This regimen which has been employed previously (Bagrov et al., 1999) induced high titers of antibodies (>1:10,000) to MBG. These antibodies had high affinity to MBG (4.7 X 10-9 to 5.5 X 10-8) and very little cross-reactivity (<<1%) to aldosterone, ouabain, digoxin, bufalin and progesterone.

Hemodynamics: Blood pressure (BP) was measured once a week by the tail cuff method (Bunag and Butterfield, 1982) in conscious, restrained rats with equipment made by IITC, Inc. (Amplifier model 229, Monitor model 31, Test chamber Model 306; IITC Life Science, Woodland Hills, CA) as described previously (Periyasamy et al., 2005). Prior to sacrifice at 4 weeks, animals had ventricular pressures determined by placement of a 2F Millar Microtip Catheter Transducer (Millar Instruments Inc., Huston, TX) into the left ventricle through a carotid insertion. Hemodynamic data were acquired at 500 Hz and stored electronically using a BioPac MP110 acquisition system and AcqKnowledge 4.7.3 software (BIOPAC Systems, Inc., Santa Barbara, CA). The values of left ventricular end diastolic pressure, systolic pressure, developed pressure, maximal velocity of rise or fall in pressure (dP/dt) and the time constant for isovolumic relaxation were determined using standard methods (Langer, 2002).
**Rat echocardiography:** At the end of 4 weeks, two-dimensional (2D) and M-mode echocardiographic studies were performed using a Philips Sonos 5500 cardiovascular ultrasound imaging system (Philips Medical Systems, Andover, MA) equipped with a 15 MHz linear transducer generously loaned to us by Dr. Philip Binkley of the Ohio State University. Parasternal long-axis and short axis views were obtained as previously described by Litwin and colleagues (Litwin et al., 1994).

**Measurement of MBG and ouabain like compound (OLC):** MBG and OLC in plasma and urine was determined at 4 weeks following extraction with C-18 columns as described previously (Priyadarshi et al., 2003). Please see http://hyper.ahajournals.org for an expanded Methods section.

**Western blot analysis:** At time of sacrifice, left ventricles were quickly dissected out, frozen in liquid nitrogen, and stored at -80°C until further analysis. Western blot analysis was performed as described previously (Kennedy et al., 2003) using SDS-PAGE gels (Ready Gel, BioRad, Richmond, CA). Please see http://hyper.ahajournals.org for an expanded Methods section.

**Measurements of oxidant stress:** Total protein carbonyl concentration of both the plasma and left ventricular homogenate was determined by ELISA using the Zentech PC Test Kit (Northwest Life Science Specialties, Vancouver, WA) (Himmelfarb and McMonagle, 2001; Himmelfarb et al., 2000). Total plasma malondialdehyde was measured spectrophotometrically using the Bioxytech MDA-586 kit (Oxis Research, Portland, OR).

**Measurement of SERCA2a activity:** To measure sarcoplasmic reticulum calcium ATPase activity which is predominantly SERCA2a activity in left ventricles of rat cardiac tissue,
the method of Simonides and Hardeveld (Simonides and van Hardeveld, 1990) was employed with minor modifications as described previously (Kennedy et al., 2003).

Miscellaneous blood analyses: Hematocrit was measured from whole blood collected in heparinized capillary tubes and spun on a micro-hematocrit centrifuge (Fisher Scientific, Chicago, IL). Plasma creatinine was determined spectrophotometrically with a colorimetric end point assay (Teco Diagnostics, Anaheim, CA). Plasma aldosterone levels were measured by ELISA (Cayman, Ann Arbor, MI). Plasma parathyroid hormone was assayed using the Rat Intact PTH ELISA kit (Immutopsics, San Clemente, CA). All analyses were performed on blood collected prior to sacrifice at 4 weeks.

Histology and Fibrosis Scoring: Please see http://hyper.ahajournals.org for an expanded Methods section.

Statistical analysis: Data presented are mean ± standard error of the mean. Please see http://hyper.ahajournals.org for an expanded Methods section.
Results:

Changes in MBG and Other Hormones:

Rats with partial nephrectomy had substantial increases in plasma [MBG] and urinary MBG excretion rates (U_{MBG}V) at 4 weeks following surgery compared with control rats (Table 1). Infusion of MBG alone induced comparable increases in plasma [MBG] and U_{MBG}V as partial nephrectomy. Immunization against MBG-BSA in partial nephrectomy animals was associated with a decrease in U_{MBG}V compared with partial nephrectomy alone. Neither plasma or urine OLC levels were different between sham and partial nephrectomy as we have previously reported (Priyadarshi et al., 2003) nor did we see a significant effect of either MBG supplementation or immunization against MBG on plasma or urine OLC levels (Table 1).

Partial nephrectomy led to marked increases in plasma creatinine and decreases in hematocrit which were not affected by immunization (Table 1). MBG infusion to sham operated rats did not significantly alter either of these measurements. Partial nephrectomy induced considerable increases in plasma aldosterone and parathyroid hormone concentrations in the plasma compared with sham operated controls (Table 1). MBG administration did not significantly increase these hormone concentrations in the plasma compared with the sham operated controls, whereas immunization against MBG did not alter these hormone concentrations compared with partial nephrectomy alone. Rats with partial nephrectomy had systemic and cardiac oxidant stress as indicated by increases in both plasma and left ventricular tissue carbonylated proteins as well as increases in plasma malondialdehyde compared with control rats, while MBG infusion alone only
produced statistically significant increases in plasma carbonylation (Table 2). Immunization against MBG in partial nephrectomy animals substantially reduced oxidant stress compared with partial nephrectomy alone (Table 2).

**Hemodynamic studies:**

Partial nephrectomy was associated with marked increases in systolic blood pressure during the 4 weeks of observation. MBG infusion alone produced some increases in blood pressure compared with control, but these increases were less than that observed with partial nephrectomy alone. Immunization against MBG did not significantly attenuate the increases in BP seen with partial nephrectomy. These data are summarized in **Figure 2a**. Echocardiographic imaging studies (see online supplemental video clips) demonstrated that partial nephrectomy animals had considerable increases in left ventricular wall thickness compared with controls (**Figure 2b-2c**). These data were consistent with the heart weight data obtained (vida infra). Left ventricular end diastolic (**Figure 2d**) and end systolic volumes (**Figure 2e**) were markedly reduced in the partial nephrectomy animals, and the calculated fractional shortening was also substantially increased (**Figure 2f**). MBG infusion was not associated with significant changes in wall thickness, chamber size, or fractional shortening compared with sham treated controls. Immunization against MBG ameliorated the echocardiographic changes noted with partial nephrectomy (**Figures 2b-2f**).

After 4 weeks the animals were anesthetized and a Millar catheter was introduced into the left ventricle in order to measure left ventricular hemodynamics. Partial nephrectomy surgery induced substantial increases in maximal velocity of rise in
pressure (dP/dt) compared with controls (Figure 3a). However, diastolic function was impaired as assessed by the ratio of maximal positive dP/dt to maximal negative dP/dt (Figure 3b), an increase in left ventricular end-diastolic pressure (LVEDP, Figure 3c) as well as the time constant for left ventricular isovolumic relaxation (Figure 3d). A similar pattern was noted in the rats subjected to MBG infusion, but only the changes in LVEDP and time constant for isovolumic relaxation achieved statistical significance.

Immunization against MBG in partial nephrectomy animals considerably attenuated the changes in maximal positive dP/dt, the ratio of maximal positive dP/dt to negative dP/dt, LVEDP, and the time constant for ventricular relaxation seen with partial nephrectomy (Figures 3a-3d).

Cardiac Morphology and Biochemistry:

Rats subjected to partial nephrectomy had marked increases in heart weight compared with control animals (Figure 4a). Although MBG infusion also resulted in significant increases in heart weight, these increases were less than that seen with partial nephrectomy. Partial nephrectomy was associated with activation of ERK (Figure 4b) and Src (Figure 4c), upregulation of skACT (Figure 4d) as well as downregulation of both the α1 and α2 isoform of the Na/K-ATPase (Figures 4e and 4f) and SERCA2a (Figure 4g). SERCA enzymatic activity was also decreased in partial nephrectomy treated animals (Figure 4h). A similar pattern of changes in protein expression was noted in rats subjected to MBG infusion. Immunization against MBG prevented or attenuated the increases in cardiac size, activation of ERK and Src, upregulation of
skACT as well as the downregulation of α2 Na/K-ATPase and SERCA2a expression and SERCA function with partial nephrectomy (Figures 4a-4g).

Partial nephrectomy resulted in marked increases in cardiac fibrosis as assessed by either semi-quantitative grade or morphometric analysis. MBG infusion produced similar histological changes as partial nephrectomy. Immunization against MBG markedly attenuated the histological changes seen with partial nephrectomy (Figures 5a-5c). Partial nephrectomy was associated with marked increases in fibronectin, while immunization against MBG markedly attenuated the changes in fibronectin (Figure 5d).
Discussion:

Patients with chronic renal failure develop cardiac disease with exceptional frequency (Middleton et al., 2001). Even mild degrees of chronic renal insufficiency are associated with marked increases in cardiovascular mortality (Henry et al., 2002). Although historically the term uremic cardiomyopathy referred to patients with a dilated cardiomyopathy not attributable to other causes, the modern concept is that patients with renal failure develop diastolic dysfunction and cardiac hypertrophy which are, at least in part, related to their renal disease (Middleton et al., 2001). The pathogenesis of this uremic cardiomyopathy has been debated (Middleton et al., 2001; Parfrey, 2001; Rigatto et al., 2002). While it is believed that extracellular volume expansion plays a key role in the development of the left ventricular hypertrophy, other factors including hyperparathyroidism, hypertension, and anemia have been proposed to be important in the pathogenesis (Middleton et al., 2001). It is interesting to note that patients with chronic renal failure also develop evidence for systemic inflammation and oxidant stress (Himmelfarb and McMonagle, 2001; Himmelfarb et al., 2000). Oxidant stress is believed to be a major pathogenic factor in the cardiovascular disease seen in renal failure (Himmelfarb et al., 2002) as well as the general population (Foley and Parfrey, 1994).

The concept that hormonal adaptations to decreases in renal function might participate in the pathogenesis of the uremic syndrome was elaborated in the 1960s. This concept is called “trade-off”; the idea is that body fluid and electrolyte homeostasis would be maintained despite renal insufficiency, but the elevated hormone levels might have deleterious consequences (Bricker, 1972). The best characterized example of “trade-
off” is the elevated parathyroid hormone levels which maintain serum phosphate levels but have deleterious effects on bone and possibly other tissues (Bricker, 1967). Interestingly, Bricker, DeWardener and others specifically postulated that an inhibitor of the plasmalemmal Na/K-ATPase which was natriuretic would accumulate in the serum and cause organ dysfunction (Bricker, 1967; de Wardener, 1996). However, our understanding of the cardiotonic steroids, previously referred to as digitalis-like substances, has undergone tremendous change. For one, specific chemicals have been identified and characterized. However, perhaps more importantly, focus has shifted from the pharmacological effect of these cardiotonic steroids on the enzymatic function of the Na/K-ATPase to the signaling that occurs through this system. Specifically, it has been clearly demonstrated that cardiotonic steroids initiate a signal cascade which is mediated through Src, Ras, reactive oxygen species and ERK and induce endocytosis of the plasmalemmal Na/K-ATPase (Haas et al., 2000; Huang et al., 1997; Kometiani et al., 1998; Liu et al., 2000; Xie et al., 1999). This signal cascade occurs in cell free systems and requires the Na/K-ATPase to be in caveolae in order to proceed (Liu et al., 2005; Liu et al., 2003). Cardiotonic steroid signaling through the sodium pump causes well described changes in gene expression which can be blocked by anti-oxidants (Liu et al., 2000; Priyadarshi et al., 2003; Xie et al., 1999).

The purpose of the current study was to examine whether this signaling by cardiotonic steroids through the Na/K-ATPase, which has been extensively characterized in vitro, actually plays a significant role in an in vivo model of uremic cardiomyopathy. Our observations can be summarized as follows. First, we observed that partial
nephrectomy was associated with virtually all of the molecular and physiological features of clinical uremic cardiomyopathy. Specifically, we found that animals subjected to partial nephrectomy developed systemic oxidant stress along with alterations of diastolic function quite consistent with that seen in patients afflicted with chronic renal failure (Harnett et al., 1994; Himmelfarb et al., 2002). This was not very surprising as the rat partial nephrectomy model has been extensively studied as a model for chronic renal failure (Hostetter et al., 1981). Next, we saw that infusion of 10 µg/kg/day of MBG, which produces levels comparable to those in partial nephrectomy rats, produced almost identical increases in the plasma level of MBG to that seen with partial nephrectomy. These MBG infusions also produced a similar degree of oxidant stress as well as some of the cardiac functional and morphological alterations seen with partial nephrectomy. Although MBG infusions did not cause significant changes in our echocardiographic measurements, they did lead to significant changes in other related measures of hypertrophy and diastolic dysfunction obtained by methods which were probably more sensitive than our echocardiographic measurements. Specifically, left ventricular catheterization revealed significant increases in both LVEDP and tau in MBG supplemented animals. Increases in heart weight and skeletal muscle actin expression were also noted in the MBG supplemented group. Third, we found that both partial nephrectomy and MBG administration induced a significant and substantial amount of cardiac fibrosis in the rat. Progressive fibrosis is believed to play a seminal role in the progression of renal failure in this model (Border and Noble, 1994), and the markedly elevated levels of aldosterone have been implicated in this process (Greene et al., 1996).
Of course, the potential role of aldosterone in the pathogenesis of cardiac fibrosis has received intense interest in the wake of the clinical findings reported in the RALES study (1996). Although plasma aldosterone concentrations were quite high in the partial nephrectomy model, we found that neither MBG infusion nor immunization against MBG appeared to alter these concentrations. Moreover, the antibody formed in rats in response to immunization against MBG did not react with aldosterone. It is interesting to note that aldosterone-induced fibrosis only appears to develop in settings where salt loading and volume expansion also occur (Young and Funder, 2002), and these settings are likely to have increased circulating concentrations of MBG (Fedorova et al., 1998; Fedorova et al., 1998). To be sure, we noted considerable cardiac fibrosis in our renal failure model as demonstrated by histological analysis and fibronectin expression. Fourth and perhaps most important, we observed that active immunization against MBG was associated with very substantial attenuation of cardiac hypertrophy, cardiac fibrosis and the oxidant stress state. The decreases in cardiac expression of SERCA2a, as well as SERCA enzymatic activity seen with partial nephrectomy, were also markedly attenuated by the active immunization. Although MBG infusions were associated with increases in blood pressure, it is noteworthy that active immunization against MBG did not substantially attenuate the hypertension. We expect that this underscores the greater importance of other factors (e.g., renin-angiotensin-aldosterone activation) in the pathogenesis of hypertension in our model.

Ferrandi and coworkers reported that longer term infusions of ouabain (15 µg/kg/day X 18 weeks) produced hypertension and cardiac hypertrophy in rats (Ferrandi
et al., 2004). They also found that these effects of ouabain could be prevented by concomitant administration of an experimental molecular antagonist to ouabain, PST 2238. This is particularly interesting in light of the recent report demonstrating that increases in brain ouabain trigger increases in peripheral MBG concentrations in the setting of salt loading (Fedorova et al., 2005).
Perspectives:

Taken together, our data strongly supports an important role for MBG in the pathogenesis of experimental uremic cardiomyopathy in the rat. This is extremely interesting in the light that volume expansion appears to play a key role in cardiac hypertrophy seen with renal failure and MBG concentrations are increased with volume expansion. Our finding that MBG is also associated with fibrosis in our model allows one to speculate that some cross talk between MBG and aldosterone in the pathogenesis of cardiac fibrosis may be possible. If MBG is also found to be important in clinical uremic cardiomyopathy, therapy targeted against MBG signaling could potentially have clinical application.

Acknowledgements:

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Literature Cited:


37. Effectiveness of spironolactone added to an angiotensin-converting enzyme inhibitor and a loop diuretic for severe chronic congestive heart failure (the


Figure Legends:

Figure 1. Schematic depicting sodium pump signaling in cardiac myocytes. In the presence of a cardiotonic steroid, Na/K-ATPase is converted to a signal transducer which complexes with Src and the EGFR. A signal cascade is initiated which depends on Ras and results in the generation of reactive oxygen species (ROS) and activation of ERK. This, in turn, leads to altered gene expression including decreases in SERCA expression as well as alterations in calcium cycling. Data supporting this schematic summarized from references (Haas et al., 2000; Huang et al., 1997; Kometiani et al., 1998; Liu et al., 2000; Xie et al., 1999).

Figure 2. MBG produces functional and anatomic changes consistent with cardiac hypertrophy. (a) Systolic blood pressure 4 weeks after sham operation (Sham, n=8), partial nephrectomy (PNx, n=8), MBG infusion (MBG, n=10) or immunization against MBG prior to partial nephrectomy (PNx-IM, n=8). (b) Representative M mode echocardiograms in the four groups of rats (c) Posterior wall thickness, (d) left ventricular end diastolic diameter, (e) left ventricular end systolic diameter, and (f) fractional shortening 4 weeks after Sham (n=8) PNx (n=10), MBG (n=9), or PNx-IM (n=16). * P < 0.05 vs. Sham; ** P < 0.01 vs. Sham; # P < 0.05 vs. PNx, ## P < 0.01 vs. PNx.

Figure 3. MBG produces hemodynamic changes consistent with diastolic dysfunction. (a) Maximal rate of pressure change (+dP/dt), (b) ratio of +dP/dt to minimal rate of pressure change (i.e., most negative rate of pressure change, -dP/dt) (c) left ventricular end diastolic pressure (LVEDP), and (d) time constant of isovolumic relaxation (τ) 4
136 weeks after sham operation (Sham, n=14), partial nephrectomy (PNx, n=15), MBG infusion (MBG, n=12) or immunization against MBG prior to partial nephrectomy (PNx-IM, n=14). * P < 0.05 vs. Sham; ** P < 0.01 vs. Sham; # P < 0.05 vs PNx, ## P < 0.01 vs. PNx.

Figure 4. MBG produces changes in cardiac morphology and protein expression consistent with experimental uremia. (a) Heart weight to body weight (HW/BW) ratio 4 weeks after sham operation (Sham, n=18), partial nephrectomy (PNx, n=20), MBG infusion (MBG, n=20) or immunization against MBG prior to partial nephrectomy (PNx-IM, n=18). (b) Extracellular signal-related kinase (ERK-1, p44) activation in the left ventricular cardiac homogenate 4 weeks after Sham (n=15), PNx (n=14), MBG (n=7) or PNx-IM (n=7). Gels were loaded with 50 µg left ventricle homogenate protein. Representative active and total ERK blots shown. (c) Src (Src pY^418) activation in the left ventricular cardiac homogenate 4 weeks after Sham (n=15), PNx (n=13), MBG (n=10) or PNx-IM (n=6). Gels were loaded with 75 µg left ventricle homogenate protein. Representative active and total Src blots shown. (d) skeletal muscle actin (skACT) (e) Na/K-ATPase α1 (f) Na/K-ATPase α2 and (g) SERCA2a expression 4 weeks after Sham (n=15), PNx (n=13), MBG (n=10) or PNx-IM (n=6). Gels for d-g were loaded with 20 µg left ventricle homogenate protein. (h) SERCA2a enzymatic activity in the left ventricular cardiac homogenate 4 weeks after Sham (n=8), PNx (n=6), MBG (n=8) or PNx-IM (n=8). The bar graphs for Western blot data summarize densitometry analysis of the blots. ** P < 0.01 vs. Sham, * P < 0.05 vs. Sham, # p< 0.05 vs PNx, ## p< 0.01 vs PNx.
Figure 5. MBG induces cardiac fibrosis. (a) Representative Masson’s trichrome sections of left ventricular cardiac tissue 4 weeks after sham operation (Sham), partial nephrectomy (PNx), MBG infusion (MBG) or immunization against MBG prior to partial nephrectomy (PNx-IM). (b) Semi-quantitative grade and (c) quantitative morphometric fibrosis scoring for trichrome slides of left ventricular cardiac sections 4 weeks after Sham (n=8), PNx (n=10), MBG (n=10) or PNx-IM (n=10). (d) Fibronectin expression and quantified data from Sham (n=9), PNx (n=9), MBG (n=9) and PNx-IM (n=9). Gels were loaded with 50 µg of left ventricle homogenate protein. * P< 0.05, ** P < 0.01 vs. Sham, # P< 0.05, ## P < 0.01 vs. PNx.
TABLE 1. Effects of MBG on Various Biochemical and Functional Parameters

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<td>11.4±1.9</td>
<td>10.6±0.9</td>
<td>11.1±1.1</td>
</tr>
<tr>
<td>Plasma Creatinine (mg/dL)</td>
<td>0.30±0.03</td>
<td>0.95±0.12†</td>
<td>0.52±0.07</td>
<td>0.95±0.13†</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>44.5±0.8</td>
<td>38.8±1.3†</td>
<td>44.6±0.9</td>
<td>41.1±0.7†</td>
</tr>
<tr>
<td>Plasma Parathyroid Hormone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(pg/mL)</td>
<td>41±6</td>
<td>126±17*</td>
<td>41±9</td>
<td>140±26*</td>
</tr>
<tr>
<td>Plasma Aldosterone (pg/mL)</td>
<td>191±55</td>
<td>1780±371†</td>
<td>322±38</td>
<td>2207±474†</td>
</tr>
</tbody>
</table>

Analyses were performed 4 weeks after sham operation (Sham, n=16), partial nephrectomy (PNx, n=20), MBG infusion (MBG, n=14) or immunization against MBG prior to partial nephrectomy (PNx-IM, n=18). * \(P < 0.05\) vs. Sham, † \(P < 0.01\)
vs. Sham, $\dagger P < 0.05$ vs. PNx, $\ddagger P < 0.01$ vs. PNx.
TABLE 2. MBG Induces Oxidant Stress In Vivo

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sham</th>
<th>PNx</th>
<th>MBG</th>
<th>PNx-IM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Carbonylated Protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(pmol/mg protein)</td>
<td>171±9</td>
<td>320±20†</td>
<td>378±15†</td>
<td>241±24‡</td>
</tr>
<tr>
<td>LV Carbonylated Protein</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>(pmol/mg protein)</td>
<td>387±23</td>
<td>541±41*</td>
<td>505±22</td>
<td>393±39§</td>
</tr>
<tr>
<td>Plasma Total Malondialdehyde</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nM)</td>
<td>399±17</td>
<td>571±49†</td>
<td>474±41</td>
<td>428±34</td>
</tr>
</tbody>
</table>

Analyses performed 4 weeks after sham operation (Sham, n=8), partial nephrectomy (PNx, n=12), MBG infusion (MBG, n=14) or immunization against MBG prior to partial nephrectomy (PNx-IM, n=18). * P < 0.05 vs. Sham, † P < 0.01 vs. Sham, ‡ P < 0.05 vs. PNx, § P < 0.01 vs. PNx.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Supplemental Methods:

Measurement of MBG and OLC: MBG and OLC in plasma and urine was determined at 4 weeks following extraction with C-18 columns as described previously; for OLC, the methodology was exactly the same (Priyadarshini et al., 2003). However, in this study, the amount of MBG was measured using an enzyme-linked immunosorbent assay (ELISA) technique. ELISA plates were coated with MBG-thyroglobulin conjugate at a dose of 60 ng/plate. Anti-MBG antiserum (V-9, titer 1:80,000) derived from rabbit was employed (100 ul/well) with an ELISA amplification kit (Invitrogen, Carlsbad, CA). C-18 column extracts were sometimes diluted so that all absorbance values could be read on the linear part of a MBG concentration curve which typically ranged from 10 pM to 1 nM.

Western blot analysis: At time of sacrifice, left ventricles were quickly dissected out, frozen in liquid nitrogen, and stored at -80°C until further analysis. Left ventricles were homogenized in imidazole buffer (25 mM, pH 7.0) containing protease inhibitors and solubilized in a buffer (pH 7.4) containing HEPES (50 mM), NaCl (50 mM), glycerol (10%), triton (1%), NP-40 (1%), deoxycholate (0.25%), and protease inhibitors. Western blot analysis was performed as described previously (Kennedy et al., 2003) using SDS-PAGE gels (Ready Gel, BioRad, Richmond, CA). Immunodetection of proteins was carried out using the following antibodies: anti-SERCA2 monoclonal antibody (mAb) (Affinity Bioreagents, Inc., Golden, CO); anti-ERK-1 polyclonal antibody (pAb) anti-Na/K-ATPase α2 pAb, anti-fibronectin mAb, and anti-phospho-ERK-1 mAb (Santa Cruz Biotechnology, Santa Cruz, CA); anti-Na/K-ATPase α1 mAb and anti-Src mAb (Upstate, Lake Placid, NY); anti-α sarcomeric actin mAb (Abcam, Cambridge, MA); anti-phospho
Src (Y418) pAb (Biosource, Camarillo, CA). The immunoreactive products for all western blots were visualized with secondary antibody conjugated to horseradish peroxidase using either SuperSignal® West Pico substrate (Pierce, Rockford, IL) or ECL Plus™ Western Blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ) and equal loading was confirmed with anti-β actin pAb or anti-α tubulin mAb (Santa Cruz Biotechnology, Santa Cruz, CA). The images of the immunoreactive products were quantified with the Molecular Analyst™ software program (BioRad Laboratories, Hercules, CA) as described previously (Kennedy et al., 2003).

**Histology and Fibrosis Scoring:** Left-ventricle sections were fixed overnight in 4% formalin, buffered with PBS, dehydrated in 70% ethanol, and transferred to xylene and embedded with paraffin. Paraffin-embedded samples were sectioned at 4 μm and Masson’s trichrome staining was performed (Shapiro et al., 1990). Semi-quantitative scoring (0-4+) of trichrome sections was assigned in a blinded fashion independently by two physicians (SV and JIS). 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 (National Institutes of Health, USA http://rsb.info.nih.gov/ij/). The amount of fibrosis was then estimated from the RGB images with a macro written by the authors (JIS) by converting pixels of the image with substantially greater (> 120%) blue than red intensity to have the new, grey scale amplitude = 1, leaving other pixels as with amplitude = 0 (**Supplemental Figure 1**). **Statistical analysis:** Data presented are mean ± standard error of the mean. Data obtained were first tested for normality. If the data did not pass the normality test, the Tukey test
(for multiple groups) or the Mann-Whitney Rank Sum test were used to compare the data. If the data did pass the normality test, parametric comparisons were performed. If more than two groups were compared, one-way analysis of variance was performed prior to comparison of individual groups with the unpaired Student’s t-test with Bonferroni’s correction for multiple comparisons (Wallenstein et al., 1980). Statistical analysis was performed using SPSS™ software.
Supplemental Figure 1.
Ouabain Decreases Sarcoplasmic Reticulum Calcium ATPase (SERCA2a) Activity in Rat Hearts by a Process Involving Protein Oxidation

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Short Title: Oxidative Modification of SERCA2a by Ouabain

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ABSTRACT:

The effect of digitalis to increase cardiac inotropy by altering calcium (Ca^{2+}) cycling is well known but still poorly understood. The studies described in this report focus on defining the effects of ouabain signaling on sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a) function in rat cardiac myocytes. First, cardiac myocytes were isolated and studied with Indo-1 fluorescence spectroscopy. Myocytes treated with ouabain (50 μM) demonstrated substantial increases in systolic (313 +/- 26 vs 260 +/- 11nM, p < 0.05) and diastolic (120 +/- 16 vs 61 +/- 10, p < 0.01) Ca^{2+} concentrations. The recovery time constant for the Ca^{2+} transient, Tau_{Ca} (τ_{Ca}), was significantly prolonged by ouabain (399 +/- 35 vs 288 +/- 26 msec, p < 0.01). Exposure to 10 μM H_{2}O_{2}, which causes a similar increase in intracellular reactive oxygen species (ROS) as 50 μM ouabain, caused a similar increase in τ_{Ca} (377 +/- 35 vs 288 +/- 35 msec, p < 0.01). Concurrent exposure to N-Acetyl Cysteine (NAC, 10 mM) or an aqueous extract from green tea (GT, 50 ug/ml) both prevented the increases in τ_{Ca} as well as the changes in systolic or diastolic Ca^{2+} concentrations. Next, using the isolated perfused rat heart model, we observed that ouabain (50 μM) induced increases in developed pressure and dP/dt as has previously been observed. In addition, the ratio of +dP/dt to –dP/dt was increased following exposure to ouabain. Co-administration of ouabain with NAC prevented these increases. Western blot analysis and coimmunoprecipitations revealed increases in both the oxidation and nitrotyrosine content of the cardiac SERCA2a in the ouabain treated hearts. Liquid chromatography/mass spectrometric analysis of cardiac immunoprecipitate confirmed that the SERCA2a protein from ouabain treated hearts had
modifications consistent with oxidative and nitrosative stress. These data demonstrate that ouabain induces marked oxidative changes of SERCA structure and function which may produce some of the associated changes in Ca$^{2+}$ cycling and physiological function.

Key Words: ouabain; calcium (Ca$^{2+}$); reactive oxygen species (ROS), cardiac signaling; sarcoplasmic reticulum Ca$^{2+}$ ATPase (SERCA2a)
INTRODUCTION

Cardiac glycosides have been used for the treatment of congestive heart failure for centuries (1). Heretofore, the molecular mechanism for these cardiac glycosides has been their effect on ion concentrations initiated through inhibition of the Na/K-ATPase (2). Our laboratory has reported that sodium pump inhibition initiates a signal cascade which does not require observable changes in intracellular sodium concentrations that might be anticipated from inhibition of the enzymatic function of the Na/K-ATPase (3). Further, we have found the generation of reactive oxygen species (ROS) as an essential part of this signal cascade (4, 5). We have also observed that the administration of either purified digitalis like substances (DLS) such as ouabain or the serum from uremic patients, which is known to contain increased concentrations of these DLS (6), produces acute diastolic dysfunction in isolated rat cardiac myocytes studied in culture (7). In separate experiments, we have also noted that cardiac myocytes isolated from rats subjected to 5/6th nephrectomy also have diastolic dysfunction which can be attributed to reduced sarcoplasmic reticulum calcium ATPase (SERCA2a) activity; interestingly, the reduction in SERCA2a activity in the 5/6th nephrectomy model occurs in concert with proportional reductions in SERCA2a mRNA and protein expression.

The reuptake of Ca\(^{2+}\) by the sarcoplasmic reticulum (SR) in myocardial cells is the major determinant of active relaxation in the rat (8). This SR Ca\(^{2+}\) reuptake is determined by several factors including the intrinsic enzymatic activity of SERCA2a and the phosphorylation status of phospholamban. Because our previous study noted that DLS altered the renormalization of Ca\(^{2+}\) following electrical stimulation in rat cardiac
myocytes very rapidly (7), we postulated that DLS signaling through the sodium pump might itself alter SERCA function, either directly or through phospholamban (PLB) phosphorylation. Because the generation of ROS is an important step in signaling through the sodium pump (4), we further speculated that these ROS might themselves be involved in the molecular alterations induced by DLS stimulation. To test these hypotheses, the following studies were performed.
METHODS

Animals: Male, Sprague Dawley rats weighing 300-350 gms were used for all studies. Animals were euthanized and hearts were removed for isolated isovolumic Langendorf perfusion, determination of SERCA2a enzymatic activity, or Western blot analysis using standard methods (7, 9, 10). In other cases, hearts were removed, and cardiac myocytes were isolated for subsequent study. All animal experimentation described in the manuscript was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals using protocols approved by the Medical College of Ohio Institutional Animal Use and Care (IACUC) Committee.

Isolation and culture of cardiac myocytes: Details of the method of isolation and culture of Ca^{2+} tolerant adult myocytes may be found in several recent reports from our laboratory (7, 9, 10). This method of isolation produced a good yield of rod-shaped (70-80%) myocytes in each of the experimental groups presented in this paper. In order to make the myocytes Ca^{2+} tolerant after isolation in Ca^{2+} free medium, the pellet was resuspended in media containing graduated increases in the concentration of Ca^{2+} from 10 μM to 1.8 mM.

Measurements of the Ca^{2+} transient: The cytosolic Ca^{2+} concentrations during contraction and relaxation (i.e., Ca^{2+} transient) were monitored with the Ca^{2+} selective fluorescent dye indo-1(Molecular Probes, Eugene, OR), employing a spectrofluorimeter (Photon Technology International, Monmouth Junction, NJ) interfaced with an inverted microscope as we have previously described (7, 9, 10). The time constant, τ_{Ca}, for recovery Ca^{2+} following electrical stimulation was performed by fitting a least square
regression line to the log transformation of the fluorescence data as described by Bassani and colleagues (8) as we have previously reported (7, 9). Measurements were performed prior to treatment and 5 minutes after ouabain exposure.

In order to determine the role of ROS in Ca\(^{2+}\) metabolism, cells were pretreated for 30 minutes with either 50 \(\mu\)g/ml aqueous green tea extract (GT) or 10 mM N-Acetyl Cysteine (NAC) before ouabain exposure. Caffeine (10mM) was used to assess SR Ca\(^{2+}\) content as well as SERCA2a and Na/Ca\(^{2+}\) exchanger activity as described by Bassani et al (8). All measurements were performed at 37°C.

**Isolated Perfused Heart:** The isolated isovolumic Langendorf preparation was performed in a similar fashion to that which we have previously described in some detail (11).

Isovolumic perfusion was performed with a perfusion pressure of 80 cm H\(_2\)O employing Krebs-Henseleit bicarbonate buffer that was oxygenated with 95% O\(_2\)-5% CO\(_2\) at 37°C (pH = 7.4). A latex balloon tipped fluid filled catheter was placed in the left ventricle through the left atrium so that left ventricular pressure could be measured with a pressure transducer. This latex balloon was filled with fluid at the start of each experiment so that the left ventricular end diastolic pressure was 8-12 mmHg. Hearts were paced with a Grass stimulator at a rate of 300 beats/min. All experiments consisted of a 30 minute period of equilibration (baseline) followed by another 30 minutes of perfusion with either Krebs-Henseleit (time control), 50 \(\mu\)M ouabain, or 50 \(\mu\)M ouabain and 10 mM NAC. For the experiments involving ouabain/NAC, hearts were pre-treated with 10 mM NAC during the equilibration phase. Hemodynamic data was acquired at 500 Hz and stored
electronically using a BioPac MP110 acquisition system and AcqKnowledge 4.7.3 software (BIOPAC Systems, Inc., Santa Barbara, CA).

**Western Blot Analysis:** At the termination of the Langendorf experiments, left ventricles were quickly dissected out, frozen in liquid nitrogen, and stored at -80°C until further analysis. Left ventricles were homogenized in 25mM imidazole buffer pH 7.0 containing protease inhibitors (Sigma, P-2714), and Western blot analysis was performed as described previously (9) using either 10% or 15% SDS-PAGE gels (Ready Gel, BioRad, Richmond, CA). Immunodetection of SERCA2a was carried out using both anti-SERCA2 monoclonal antibody (mAb) (Affinity Bioreagents, Inc., Golden, CO) and anti-SERCA2 polyclonal antibody conjugated directly to horse radish peroxidase (Bethy Laboratories). Immunodetection of PLB was obtained with anti-PLB mAb (Affinity Bioreagents, Inc.) while PLB phosphorylation sites were obtained with goat polyclonal antibodies raised against a PLB peptide phosphorylated at Ser16 (Santa Cruz Biotechnology, Santa Cruz, CA). For immunodetection of oxidatively modified proteins, 10 mg/ml of left ventricular (LV) homogenate was derivatized with either 2,4-dinitrophenyl hydrazine or a derivatization control solution and detected using the OxyBlot™ Protein Oxidation Detection kit according to the manufacturer’s protocol (Chemicon International, Temecula, CA). As a marker of reactive nitrogen species (RNS) and peroxynitrite (ONOO-), tyrosine nitration was detected using anti-nitrotyrosine mAb (Cayman, Ann Arbor, MI). The immunoreactive products for all western blots were visualized with secondary antibody conjugated to horseradish peroxidase using either SuperSignal® West Pico substrate (Pierce, Rockford, IL) or ECL
Plus™ Western Blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ). The images of the immunoreactive products were quantified with a Molecular Analyst™ software program (BioRad Laboratories, Hercules, CA) as described previously (9).

**Measurement of SERCA2a Activity:** To measure sarcoplasmic reticulum Ca\(^{2+}\) ATPase activity, which is predominantly SERCA2a activity in rat cardiac tissue, the method of Simonides and Hardeveld (12) was employed with minor modifications as described previously (9).

**ELISA analysis:** Total protein carbonyl concentration of the LV homogenate was determined by enzyme-linked immunosorbent assay (ELISA) using the Zentech PC Test Kit according to the manufacturer’s protocol (Northwest Life Science Specialties, Vancouver, WA).

**Immunoprecipitation:** Left ventricular homogenate was solubilized in radioimmune precipitation buffer containing protease inhibitor cocktail (Sigma P-2714) for 2 hours at 4°C and then centrifuged at 16,000 x g for 20 minutes to remove insoluble material. The solubilized protein fraction (1 mg/mL) was then immunoprecipitated using polyclonal anti-SERCA2a antibody (Abcam, Cambridge, MA) overnight at 4°C. Immune complexes were collected on protein G sepharose (Upstate, Lake Placid, NY) and washed four times in the immunoprecipitation buffer. The immunoprecipitate was dissolved in sample buffer, separated on 10% SDS-PAGE, and blotted with either polyclonal anti-DNP antibody (Chemicon International, Temecula, CA) or anti-nitrotyrosine mAb. To confirm the presence of SERCA2a oxidation, some samples were first derivatized with 2,4-dinitrophenyl hydrazine or a derivatization control solution and immunoprecipitated
using polyclonal anti-DNP antibody overnight at 4°C. RNS/peroxinitrite modifications were confirmed using anti-nitrotyrosine mAb overnight at 4°C. Immune complexes were processed as described above and blotted with anti-SERCA2a mAb. All gels were run in duplicate so that identical gels could be processed for mass spectrophotometric analysis as described below.

**Liquid chromatography/tandem mass spectrometric (LC-MS) analysis:** In order to obtain positive identification of SERCA as well as to investigate the presence of post-translational modifications, gel slices obtained from duplicate immunoprecipitate gels were analyzed by LC-tandem MS after in-gel proteolysis as described elsewhere (13). Briefly, after destaining the Coomassie stained gel slices with 30% methanol for 3 h at RT, they were digested with 1 mg of sequencing grade, modified trypsin (Promega) in 0.1M ammonium bicarbonate buffer (pH 8.0) for 12 h at 37°C. Peptides were extracted once each with 60% acetonitrile:0.1% trifluoroacetic acid (TFA) and acetonitrile:0.1% TFA. The extracts were pooled and concentrated down to ~15 ml using vacufuge. Two ml of the digest were separated on a reverse phase column (75 um id X 5 cm X 15 um Aquasil C18 PicoFrit column, New Objectives) using a 1% acetic acid/acetonitrile gradient system (5-75% acetonitrile over 35 min, followed by a 3 min wash with 95% acetonitrile) at a flow rate of ~250 nl/min. The peptide eluent was directly introduced into an ion-trap mass spectrometer (LCQ-Deca XP Plus, Thermofinnigan) equipped with a nano-spray ionization source. The mass spectrometer was operated on a double play mode where the instrument was set to acquire a full MS scan (400-2000 m/z) and a collision induced dissociation (CID) spectrum on the most abundant ion from the full MS
scan (relative collision energy ~30%). Dynamic exclusion was set to acquire 3 CIDS on the most abundant ion and exclude it for a further 2 min. The CID spectra were searched against a subset of non-redundant, indexed rat protein database using TurboSEQUEST search program (Thermofinnigan). Peptide hits with Xcorr and DCn of >2 and >0.2, respectively, were considered positive and verified manually. Any uninterpreted spectra were manually searched using the MS-Tag provision of Protein Prospector (http://prospector.ucsf.edu) (14).

Statistical Analysis: Data presented are mean± standard error of the mean. Data obtained were first tested for normality. If the data did not pass the normality test, the Tukey test (for multiple groups) or the Mann-Whitney Rank Sum test were used to compare the data. If the data did pass the normality test, parametric comparisons were performed. If more than two groups were compared, one-way analysis of variance was performed prior to comparison of individual groups with the unpaired Student’s t-test with Bonferroni’s correction for multiple comparisons. If only two groups of normal data were compared, the Student’s t-test was used without correction (15). Statistical analysis was performed using SPSS™ software.
RESULTS

Effect of Ouabain on Isolated Cardiac Myocyte Calcium Cycling and Contractile Function: Myocytes treated with ouabain (50 μM) demonstrated substantial increases in systolic (313 +/- 26 vs 260 +/- 11nM, Figure 1a) and diastolic Ca\(^{2+}\) (120 +/- 16 vs 61 +/- 10, p < 0.01, Figure 1b). The recovery time constant for the Ca\(^{2+}\) transient, \(\tau_{Ca}\), was also prolonged by ouabain exposure (399 +/- 35 vs 288 +/- 26 msec, p < 0.01, Figure 1c). Exposure to 10 mM H\(_2\)O\(_2\) which causes a similar increase in intracellular reactive oxygen species (ROS) as 50 mM ouabain (4) caused a similar increase in \(\tau_{Ca}\) (377 +/- 35 vs 288 +/- 35, p < 0.01, Figure 1c). Concurrent exposure to NAC (10 mM) or an aqueous extract from green tea (GT, 50 ug/ml), maneuvers we have previously used to prevent increased ROS induced by ouabain (5, 10, 16) both prevented the increases in systolic and diastolic cytosolic Ca\(^{2+}\) as well as \(\tau_{Ca}\) (Figure 1a-c). The ratio between the maximal caffeine and maximal twitch Ca\(^{2+}\) was reduced in the ouabain treated cells, indicating a reduced SR Ca\(^{2+}\) load and SERCA2a activity (Figure 2a). Finally, the \(\tau_{Ca}\) for the caffeine contractures was significantly prolonged in the cardiac myocytes exposed to ouabain (Figure 2b).

Effect of ouabain on isovolumic perfused heart function.

The effects of ouabain on left ventricular pressure are summarized in Table 1. We observed that ouabain induced increases in systolic function as assessed by developed pressure and the maximum and minimum rate of pressure development (+dP/dt and – dP/dt). Ouabain also impaired diastolic function, as assessed by the ratio of +dP/dt to – dP/dt, compared to control hearts at 30 minutes (Table1). Coadministration with NAC considerably attenuated ouabain’s effects on systolic and diastolic function (Table 1).
Effect of Ouabain and ROS on SERCA activity:

Isolated hearts treated with 50 mM ouabain showed substantially decreased SERCA enzymatic activity compared with time controls or hearts where NAC was administered concurrently with ouabain (Figure 3). Furthermore, exposure of LV homogenate to various concentrations of hydrogen peroxide demonstrated the ability of ROS to depress SERCA activity in a dose dependent manner (Figure 3).

Effect of Ouabain on SERCA2a and Other Cardiac Proteins:

To further examine the mechanisms underlying the alterations in cardiac function seen in both the cardiac myocytes and isolated perfused heart, SERCA2a expression was examined as well as the expression of total PLB and PLB phosphorylation (P-Ser\(^{16}\)-PLB). Quantification of protein density with western blotting, however, showed no significant change in the hearts treated with 50 mM ouabain compared with time controls with regard to SERCA2a, PLB, or P-Ser\(^{16}\)-PLB (Figure 4 A-E).

It was interesting to note, however, that SERCA2a immunoblots of ouabain treated hearts showed additional bands below the expected 110 Kda band (Figure 5a). Given the role of ROS in DLS induced signal transduction and the biochemical and physiologic data pointing to decreased SERCA2a Ca\(^{2+}\) handling in this setting, we decide to investigate if the additional bands were peptide products generated from oxidation of the SERCA2a protein. Further, as ouabain has been shown to increase levels of nitric oxide (17, 18) which in combination with ROS can yield potent peroxinitrite mediated protein modification, we also investigated the presence of nitrotyrosine as a marker of such modifications.
To these ends we first assessed the oxidation status of the whole LV homogenate using an ELISA immunoassay for carbonylated proteins. Total carbonylation was increased with ouabain treatment (0.44 nmol/mg protein) vs control and NAC+ ouabain hearts (0.21 and 0.13 nmol/mg protein respectively, p<0.05). Next, using a carbonylation immunoblot, we noted that acute ouabain administration did indeed increase both the total oxidation of cardiac proteins (Figure 5 b) and in particular the 110Kda region containing SERCA2a (Figure 5c). We also noted that ouabain administration increased tyrosine nitration, a marker of peroxynitrite modifications, of cardiac proteins (Figures 5 d) including the same 110Kda region containing SERCA2a (Figures 5 e).
To carry this analysis further, we performed several coimmunoprecipitations to determine positive identification of the oxidative/nitrosative status of SERCA2a. In the first coimmunoprecipitation we immunoprecipitated SERCA2a from the LV homogenate and immunoblotted for DNP-derivatized proteins while immunoprecipitating DNP-derivatized proteins from the LV homogenate and immunoblotting for SERCA2a. Not only did this reveal an increase in immunodetected products in the ouabain treated hearts, but also a similar pattern of additional bands was noted as in the immunoblots (Figures 6a and 6b). We also immunoprecipitated SERCA2a from the LV homogenate and immunoblotted for tyrosine nitration while immunoprecipitating nitrotyrosine proteins from the LV and immunoblotting for SERCA2a. This coimmunoprecipitation also revealed an increase in immunodetected products in the ouabain treated hearts (Figure 6c and 6d).

Finally, in order to verify oxidative and nitrosative modifications of SERCA2a, we performed LC-MS analysis of duplicate SDS-PAGE gels from the experiments outlined above. Here, we obtained positive identification of SERCA2a peptides after the immunoprecipitations for carbonylation, nitrotyrosine, or SERCA2a itself from ouabain treated hearts (Figure 7). In addition, LC-MS analysis revealed formation of a disulfide bond between Cys$^{344}$ and Cys$^{349}$ of SERCA2a.
DISCUSSION

Digitalis has played an important role in the treatment of heart failure for many years. Several laboratories, including our own, have observed that digitalis and DLS may have acute effects on ventricular relaxation (7, 19-22); our group has connected these changes in diastolic function to alterations in Ca\textsuperscript{2+} cycling (7). Although the effect of digitalis to alter Ca\textsuperscript{2+} cycling has been ascribed to changes in cytosolic sodium resulting from the inhibition of the plasmalemmal Na/K-ATPase, recent data from our laboratory and others has demonstrated that DLS can signal through the sodium pump without observable changes in cytosolic sodium (3, 16, 23-26) or even in a cell free system. Our previous work in this area also established that increases in ROS play an important role in the DLS signaling process (3-5). Of interest, it appears that ROS may also inhibit the Na/K-ATPase and initiate its signaling function, thus creating a potential positive feedback loop (27). Moreover, administration of antioxidants can attenuate both the production of ROS and the cardiac effects of DLS (5, 10, 16). An extensive body of literature has established SERCA2a as the logical focus to better understand alterations in Ca\textsuperscript{2+} cycling produced by ouabain (28-31).

While the mechanisms by which oxidative stress may induce functional modifications to ouabain treated hearts are not clear, multiple mechanisms may be operant. The role of ROS such as O\textsubscript{2}-, H\textsubscript{2}O\textsubscript{2}, and OH\textsuperscript{-} has been well established in the structural and functional pathogenesis of models such as ischemia/reperfusion. In fact, oxygen radicals have been implicated in depressing the SERCA Ca\textsuperscript{2+} handling and gene expression in the ischemic/reperfused heart (32). It is possible that ROS could act post-
transcriptionally but pre-translationally to decrease SERCA2a mRNA half-life and thus expression (33). Alternatively, ROS could act transcriptionally to decrease SERCA2a expression by acting on the SERCA2a gene. This idea seems to be supported by various models which have indirectly linked ROS to transcriptional control of SERCA2 (34-37). However, as we did not observe significant changes in the expression of either SERCA or PLB, these effects appear to be either inoperative or irrelevant in our model.

The major ROS responsible for protein oxidation are $O_2^-$, $H_2O_2$, and $OH^-$. $O_2^-$ is generated mainly from mitochondrial oxidation and enzymatic sources including NADPH oxidase, xanthine oxidase, and the endothelial nitric oxide synthase enzyme (38). In the heart, mitochondrial and cytoplasmic superoxide dismutase catalyzes the dismutation of $O_2^-$ into $H_2O_2$ and oxygen. Catalase and glutathione peroxidase hydrolyze the $H_2O_2$ generated from this reaction. Antioxidants such as NAC and green tea catechins can act to lower the concentration of ROS and prevent lipid peroxidation by acting as effective donors for the reduction of many free radical species (39-41).

Our results showing that ouabain impairs active relaxation in both whole hearts and isolated myocytes are in agreement with previous reports from our laboratory (7) and others (42-45). The improvement in active relaxation seen in the NAC + ouabain treated hearts and in $Ca^{2+}$ renormalization following stimulation in the isolated myocytes with NAC+ouabain and GT+ouabain further supports the involvement of ROS in ouabain induced alterations in $Ca^{2+}$ cycling. The impaired active relaxation observed in our isolated perfused heart studies does not appear to be explained by an increase in the PLB/SERCA ratio (46) or by a decrease in the ratio of P-Ser16 to PLB ratio (47, 48) as
these ratios remained unchanged in our model. Furthermore, the fact that the expression of both SERCA2a and PLB was unchanged contrasts with the decreased expression of both of these proteins after similar exposure to ischemia/reperfusion injury (49).

As ROS convert some amino acid residues into derivatives of aldehydes and ketones, the accumulation of carbonyl groups has proven to be a useful measure of oxidative protein modification associated with various conditions of oxidative stress (reviewed in (50) and (51)). In this study we have observed the effects of treatment with NAC and GT on both the diastolic dysfunction and depressed SERCA function induced by ouabain treatment. Proteomic analyses by western blotting, immunoprecipitation, and LC-MS seems to confirm that decreased SERCA activity is accompanied by oxidative modifications of the SERCA protein. It is also possible that the protective effect of either the NAC or GT treatments with respect to changes in SERCA function may be due to the prevention of lipid peroxidation as oxygen radicals have been shown to promote lipid peroxidation in SR membranes (52).

Peroxynitrite, formed by the combination of super oxide anion and nitric oxide (53), mediates potent and preferential oxidation of thiols such as cysteine (54). Ouabain treatment lends itself to peroxynitrite formation as ouabain has been demonstrated to increase both levels of nitric oxide (17, 18) and ROS (4, 55). Oxygen radicals have also been shown to modify SERCA activity (56-59). Thiol containing peptides present particularly sensitive targets for oxidative modifications (60, 61). As SERCA2a contains 29 cysteine residues, it is very possible that the ouabain induced changes in SERCA function may be due to the effect on free sulfhydryl groups. Indeed, LC-MS analysis of
ouabain treated hearts identified oxidation of cysteines at positions 344 and 349 with the formation of a disulfide bond (Figure 9). Interestingly, other groups have found that a peroxinitrite mediated process is capable of oxidizing free cysteines to disulfides in the SERCA1 isoform (62) and that the same Cys\textsuperscript{344} and Cys\textsuperscript{349} residues are effected (63). Furthermore, Viner and coworkers noted that modification of Cys\textsuperscript{349} is sufficient to significantly decrease SERCA1 activity in skeletal muscle (64) and that the SERCA2a isoform was up to four times more susceptible to oxidative modification by peroxynitrite than SERCA1(65). The significant trend toward increased levels of nitrotyrosine in ouabain treated hearts warrants closer examination of this potentially potent modification.

In any event, the fact that ouabain treated hearts and myocytes had increased levels of carbonylation and tyrosine nitration, decreased SR Ca\textsuperscript{2+} content, lower SERCA2a activity, and slower diastolic function points toward improved SR Ca\textsuperscript{2+} handling with antioxidant therapy. The finding that all of these effects were reversed with antioxidant therapy lends support to the novel role oxygen radicals may play in mediating the effects of ouabain. The role of ROS are further supported by the finding that LV homogenate exposed to H\textsubscript{2}O\textsubscript{2} demonstrated decreased SERCA activity in a dose dependent fashion similar to the decreases observed with ouabain treatment. Furthermore oxidative modifications present an attractive signaling mechanism for changes in Ca\textsuperscript{2+} handling as a variety of these modifications, including even that of peroxynitrite (66, 67), are potentially reversible and thus assume a dynamic physiological role.

Interestingly, systolic as well as diastolic function was altered by antioxidant administration; in fact, all of the increases in inotropy a well as systolic and diastolic Ca\textsuperscript{2+}
concentrations induced by ouabain could be prevented by administration of antioxidants. These data are seemingly at odds with recent papers linking increases in SERCA activity to improved systolic function as well as a large body of work, some from our own laboratory, linking decreases in SERCA activity with decreased inotropy in chronic models (9, 68-70). Some insight into this issue may derive from the recent paper from Teucher and colleagues who performed various levels of transfection of SERCA into rabbit myocytes. These workers found that whereas active relaxation was improved in a dose dependent manner, the lower transfection dose of SERCA increased systolic function whereas the higher amount actually attenuated systolic function (68). As the rat depends more on SERCA for active relaxation than most other species including the rabbit (8), we propose that modest impairment of SERCA in this system also explains the increases in inotropy. This concept is supported by our previous work in hearts of animals subjected to experimental renal failure where we observed impaired SERCA activity correlated with increases in systolic and diastolic Ca\textsuperscript{2+}. Here we should also mention that the prolonged time constant for recovery after caffeine treatment leaves open the possibility for involvement of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger; a line of investigation we did not pursue in these studies given Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger’s diminished contribution to Ca\textsuperscript{2+} handling in the rat (8). While Nishio and colleagues determined that the cellular effects of ouabain were independent of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (71), this same group has implicated a role for the ryanodine receptor in ouabain’s inotropic effects (72). It is clear that additional work examining the effects of ouabain and the ROS/RNS generated by
ouabain signaling on this and other proteins important in \( \text{Ca}^{2+} \) cycling and contractile function will be necessary to completely examine this important issue.

In summary, these data suggest that ouabain impairs SERCA activity in a ROS/RNS dependent manner. Oxidative and nitrosative modifications of the SERCA protein suggest molecular mechanisms by which ouabain may alter cardiac \( \text{Ca}^{2+} \) cycling and physiological function.

ACKNOWLEDGEMENTS:

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LITERATURE CITED


isoforms of Na+/K(+) -ATPase expressed in baculovirus-infected insect cells. 
*Biochem Biophys Res Commun* 207:155-159.


Table 1: Hemodynamics and contractile function of isolated hearts.

<table>
<thead>
<tr>
<th></th>
<th>Control Hearts</th>
<th>50 µM Ouabain Treated Hearts</th>
<th>10 mM NAC + 50 µM ouabain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>30 min</td>
<td>Baseline</td>
</tr>
<tr>
<td>Systolic (mmHg)</td>
<td>126±9</td>
<td>119±7</td>
<td>125±5</td>
</tr>
<tr>
<td>Diastolic (mmHg)</td>
<td>10±0.4</td>
<td>10±1.3</td>
<td>10±0.4</td>
</tr>
<tr>
<td>Developed Pressure (mmHg)</td>
<td>115±9</td>
<td>110±7</td>
<td>115±5</td>
</tr>
<tr>
<td>+dP/dt (mmHg/s)</td>
<td>2365±193</td>
<td>2292±197</td>
<td>2647±104</td>
</tr>
<tr>
<td>-dP/dt (mmHg/s)</td>
<td>2155±183</td>
<td>2106±180</td>
<td>2398±124</td>
</tr>
<tr>
<td>+dP/dt to -dP/dt ratio</td>
<td>1.11±0.02</td>
<td>1.09±0.02</td>
<td>1.11±0.02</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM for 9-16 rats in each group. **p < 0.001 vs baseline; ## p < 0.001 vs. Control hearts, 30 minutes; †p < 0.05 vs. Ouabain hearts, 30 minutes. ††p < 0.001 vs. Ouabain hearts, 30 minutes. +dP/dt, rate of pressure development, -dP/dt, rate of pressure decline.
FIGURE LEGENDS

Figure 1: Effect of ouabain (O), hydrogen peroxide (H), Green Tea Extract plus ouabain (GT+O) and N-acetyl-cysteine plus ouabain (N+O) on A) systolic calcium, B) diastolic calcium and C) calcium removal (tau) in cultured adult rat cardiac myocytes. Data are mean ± SEM from 6 different preparations. ** p < 0.01 vs. Baseline.

Figures 2: Effect of ouabain and NAC on both A) maximal caffeine/twitch calcium ratio and B) recovery of the calcium transient after administration of 10 mM caffeine in cultured adult rat cardiac myocytes. Data are mean ± SEM from 7 different preparations. ** p < 0.01, ## p < 0.0001 vs. control.

Figure 3: SERCA enzymatic activity in rat hearts after 30 min treatment with Krebs (C, n=9), 50 µM ouabain (O, n=6), 10 mM NAC and 50 µM ouabain (N+O, n=5), 1µM H_2O_2 (n=5), 10µM H_2O_2 (n=5), or 100 µM H_2O_2 (n=5). Data are presented as mean ±SEM, * p < 0.01, ** p < 0.0001.

Figure 4: A-C: Immunoblots demonstrating levels of A) SERCA2a, B) Phospholamban (PLB), and C) phosphorylated PLB at serine-16 (P-Ser^{16} PLB) in rat hearts after 30 min langendorf perfusion with Krebs (C, n=5), 50 µM ouabain (O, n=8) or 10 mM NAC and 50 µM ouabain (N+O, n=5), D-E: Relative ratios of D) PLB to SERCA2a, E) P-Ser^{16} PLB to total PLB levels. Data are presented as mean ±SEM, * p < 0.05.

Figure 5. Acute ouabain administration increases oxidation of cardiac proteins after 30 min langendorf perfusion with Krebs (C, n=5), 50 µM ouabain (O, n=8) or 10
mM NAC and 50 µM ouabain (N+O, n=5). A) Immunoblot for SERCA2a demonstrating additional bands in Ouabain treated left ventricles, B) immunoblot for carbonylated proteins demonstrating increased oxidation status in Ouabain treated left ventricles, C) densitometric quantitation of 110Kda region from carbonylation immunoblot, D) immunoblot for nitrotyrosine, E) densitometric quantitation of 110Kda region from tyrosine nitration immunoblot. Data are presented as mean ±SEM, * p < 0.05.

Figure 6. Acute ouabain administration increases oxidation (carbonylation) and tyrosine nitration of cardiac proteins. A) Immunoprecipitation of SERCA2a with immunoblot against DNP, B) immunoprecipitation of carbonylated proteins (derivatized with DNP) with immunoblot against SERCA2a, C) immunoprecipitation of SERCA2a with immunoblot against nitrotyrosine, D) immunoprecipitation of nitrotyrosine with immunoblot against SERCA2a.

Figure 7. SERCA2a protein sequence highlighting identified peptides from LC-MS analysis of ouabain treated LV homogenate, carbonylated (DNP-derivatized) and nitrotyrosine immunoprecipitate. Underlined peptides identified from 110 Kda band of SDS-PAGE of ouabain treated LV homogenate, while peptides in boldface type were identified from the specified duplicate immunoprecipitate gels.
Figure 1a.

![Bar chart showing systolic Ca$^{2+}$ (nM) over time.](image)

**Y-axis:** Systolic Ca$^{2+}$ (nM)

**X-axis:** Time (min): 0, 5

**Legend:**
- O
- H$_2$O$_2$
- GT+O
- N+O

Figure 1b.

![Bar chart showing diastolic Ca$^{2+}$ (nM) over time.](image)

**Y-axis:** Diastolic Ca$^{2+}$ (nM)

**X-axis:** Time (min): 0, 5

**Legend:**
- O
- H$_2$O$_2$
- GT+O
- N+O

*Note: The chart includes statistical significance indicated by **.***
Figure 1c.
Figure 2a.

![Graph showing Max Caffeine to Max Twitch $[Ca^{2+}]_{i}$ Ratio across C, O, and N+O conditions.]

Figure 2b.

![Graph showing $T_{Ca^{2+}}$ Caffeine Contracture (sec) across C, O, and N+O conditions. Notations ** and ## indicate significant differences.]
Figure 4a.

![SERCA2a](image)

Figure 4b.

![PLB](image)
Figure 4c.

![Image of P-Ser\textsuperscript{16} PLB and bar chart showing fraction of control for C, O, and N+O conditions.]

Figure 4d.

![Image of PLB to SERCA2a ratio and bar chart showing fraction of control for C, O, and N+O conditions.]

186
Figure 4e.

P-Ser\textsuperscript{16} PLB to PLB ratio

Fraction of Control

C  O  N+O
Figure 5a.

**WB: SERCA2a**

![Image of WB: SERCA2a with labeled bands at 118 Kda and 83 Kda.]

Figure 5b.

**WB: Carbonylation (DNP)**

![Image of WB: Carbonylation (DNP) with labeled bands at 201 Kda, 120 Kda, 100 Kda, 56 Kda, 38 Kda, and 30 Kda.]

188
Figure 5c.

Carbonylation of 110 KDa region

![Bar graph showing carbonylation levels in different conditions.]

Figure 5d.

WB: Nitrotyrosine

![Western blot image showing nitrotyrosine levels at 118 KDa and 83 KDa.]

Figure 6e.

Tyrosine nitration of 110 KDa region

![Bar graph showing tyrosine nitration levels in different conditions.]
**Figure 6a.**

**IP: SERCA2a**

**WB: Carbonylation (DNP)**

<table>
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<th></th>
<th>C</th>
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<tbody>
<tr>
<td>118 Kda</td>
<td></td>
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<tr>
<td>83 Kda</td>
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**Figure 6b.**

**IP: Carbonylation (DNP)**

**WB: SERCA2a**

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<td></td>
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</tr>
<tr>
<td></td>
<td></td>
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<td>Band 3</td>
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**Figure 6c.**

**IP: SERCA2a**

**WB: Nitrotyrosine**

<table>
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</tr>
<tr>
<td>83 Kda</td>
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</tbody>
</table>
Figure 6d.

**IP:** Nitrotyrosine  
**WB:** SERCA2a
Figure 7.

**SERCA2a Protein Sequence**

MENAHTKVEEVGLHFGVNESTGLSEQVKKLKERWSNELPAEEGKTLLELVEIQFEDLLVRILLACISFVLAWEFEGITAFVEPVFLILVA
NAIYGVWQ4ERNAENAI4EALKYPEMGKVYRQDKSVQRIKATDI4PGDI
VEIAVGDKVPA4IRTSKSTTLRVDQ5ILTGE5SVIKHTDPV4DPRAV
NQDKKNNMLFSGTNIAAGKAMGVVATGVNEIGKIRD4MEVATEQERTPLQ
QLDE4FEQSLKSVLSCIAWVIIHGFN4P3HWSWIRGAIYF4IAV
ALAVAAIPEGLPAVIT4CLALGTRMKANIVRSLPSVE4LGC4T5VICS
DKTGTLLTNQMSVCRMFLDKEGDTCSNLFTITGSTAPIGEVQDKDK
PVKCHQYDGVL4ELAT4LCNDSALDYNEAKGVYEKVGEAT4LALTCLVE
KM4NFD4ELKGLSKIERANACNSVIQLMKKEFTLESDRKSMSVYCTP
NKP4RTSMKMFVKGAP4EGVIRDCHTIRVGST4VPMP4TVKGK45MSVIRE
WG4S4GD4TLRCLALATHDNP4LRR4E4MHLEDS4N4FIYETN4LVG4C4G4MLD
PP4RIE4V4ASSVKLCRQAI4IRVIMIT4DNKGTAVAICRRIGIF4G4Q4D4ED4V4T5K
AFT4G4RE4D4F4L4PS4AQ4RD4ACLNARCFARVEPSH4SK4IV4E4FLQ4SF4DE44TAMT
GDG4V4N4D4PAL4K4SE4IG4AMG4S4GT4AV4AKTASE44MV4LADDNST4I4VA4E4E4GR
AIYNM4K4Q4F4IR4Y4L4I4SS4NV4G4EVC4IF4LA4L4G4F44E4AL4P4VQ4LL4W4V4N4L4T4D4G
A4AW4W4F44A4DG4P4R4VS4F4Y4QL4SH4F4LQ4CK4ED4NP4D4F4E4GD4C44F4E4SP4Y4P4MT4M4L
SV4L4V4T4I4E4MC4N4A4L4N4S4L44N4S4L4R4M4P4P4W4N4W4L4V4S4C4L4M4S4L4H4F4L44Y4V4E4
450
100
192

1) **VDQ5ILTGE5SVIK**: peptide 175-189 identified from post nitrotyrosine-IP, Band 3 of post DNP-IP, and post SERCA2a-IP of ouabain treated heart (see figure 7)

2) **NMLFSGTNIAAGK**: peptide 206-218 identified from post SERCA2a-IP of ouabain treated heart (see figure 7)

3) **AMGVV4ATGVNEIGK**: peptide 219-234 identified from Band 1 post DNP-IP and from post SERCA2a-IP ouabain treated heart (see figure 7)

4) **SLPSVE4LGC4T5VICS**: peptide 335-352 containing cysteine-cysteine disulfide bond, identified from top (110Kda) band of ouabain treated heart (see figure 6a)

5) **IGIF4G4Q4D4ED4V4T5K**: peptide 638-650 identified from post SERCA2a-IP of ouabain treated heart (see figure 7)
SUMMARY

Patients with chronic renal failure develop cardiac disease with exceptional frequency. Even mild degrees of chronic renal insufficiency are associated with marked increases in cardiovascular mortality. Although historically the term uremic cardiomyopathy referred to patients with a dilated cardiomyopathy not attributable to other causes, the modern concept is that patients with renal failure develop diastolic dysfunction and cardiac hypertrophy which are, at least in part, related to their renal disease (Harnett et al., 1995; Harnett and Parfrey, 1994 a, b; Harnett et al., 1988; Parfrey et al., 1996; Parfrey and Harnett, 1994 a, c). It is interesting to note that patients with chronic renal failure also develop evidence for systemic inflammation and oxidant stress (Himmelfarb and McMonagle, 2001 a, b; Himmelfarb et al., 2000). Oxidant stress is believed to be a major pathogenetic factor in the cardiovascular disease seen in renal failure as well as the general population (Foley and Parfrey, 1994).

Ischemic nephropathy is a significant yet poorly understood contributor to chronic renal failure and ESRD. Thus, we first investigated a clinical population of patients with ischemic renal disease in order to examine the implications of stabilizing or improving renal function as it related to the effects on cardiac morbidity and mortality. Our findings suggest that renal insufficiency is a significant predictor of both adverse events and cardiovascular mortality in this population.
The relationship between renal insufficiency and adverse events is strengthened by the significant decline in event free survival with advancing renal insufficiency observed in this series. Similarly, the relationship between renal insufficiency and cardiovascular and renal mortality is strengthened by the significant decline in survival with advancing renal insufficiency. Furthermore, the prevalence of coronary artery disease was equivalent across the four categories of renal function by which we classified our patients suggesting that advanced atherosclerosis is not the primary causative factor of mortality and events in this group. Importantly, this relationship between renal insufficiency, mortality, and event free survival was not attributable to the presence of coronary artery disease, diabetes or other confounding factors as evidenced by our multivariate models.

While declining renal function was associated with lower event free survival, there is an encouraging trend that improvement in renal function was associated with better survival. Moreover, there is a significant linear trend of increased adverse events as well as all cause and cardiovascular mortality in patients with decreased baseline CrCl.

We then turned to the partial nephrectomy model in the rat as this model has been used extensively to simulate experimental uremia in order to study the cardiac abnormalities that accompany renal failure. Employing this model, we examined the molecular mechanisms by which chronic renal failure contributes to diastolic dysfunction and altered calcium handling. We observed that chronic renal failure induced by partial (5/6th) nephrectomy was associated with marked increases in cardiac size, a phenomenon that
could not be accounted for only by the hypertension associated with this model. We also noted characteristic changes in message expression quite similar to that seen in ouabain induced cardiac hypertrophy in vitro. Specifically, we saw increases in the transcription of ANP and skACT, which appears to accompany all pressure overload type cardiac hypertrophy as well as decreases in the α2 isoform of Na⁺/K⁺ ATPase.

Regarding myocyte function, we found that the myocytes isolated from the hearts of rats bearing remnant kidney demonstrated both systolic and diastolic dysfunction in vitro. This was in contrast to the heart cells isolated from the aortic clip rats which demonstrated grossly normal function and calcium cycling after 4-6 wk of aortic banding. The systolic dysfunction seen in the heart cells from the remnant kidney rats was strikingly similar to that observed when heart cells are isolated from rats with congestive heart failure from a variety of causes including aortic constriction. In particular, we noted marked calcium insensitivity in these heart cells with the diastolic calcium value substantially elevated compared with sham treated rats. In addition to this systolic dysfunction, we also observed substantial diastolic dysfunction characterized by delayed recovery of length in parallel with a longer time constant τ describing calcium recovery following stimulation. In addition, we noted decreased levels and activity of SERCA2a and increased levels of NCX-1.

Next, we postulated that increases in circulating MBG were both necessary and sufficient to induce the systemic oxidant stress state and the anatomic and functional cardiac
changes seen with experimental uremia. The purpose of this study was to examine whether this signaling by cardiotonic steroids through the Na/K-ATPase, which has been extensively characterized in vitro, actually plays a significant role in an in vivo model of uremic cardiomyopathy.

Several of our observations were quite interesting. First, we observed that partial nephrectomy was associated with virtually all of the molecular and physiological features of clinical uremic cardiomyopathy including systemic oxidant stress and diastolic dysfunction quite consistent with that seen in patients afflicted with chronic renal failure. Next, we saw that infusion of 10 µg/kg/day of MBG produced almost identical increases in the plasma level of MBG to that seen with partial nephrectomy. These MBG infusions also produced a similar degree of oxidant stress as well as most of the cardiac functional and morphological alterations seen with partial nephrectomy. Third, we found that both partial nephrectomy and MBG administration induced a significant and substantial amount of cardiac fibrosis in the rat. Fourth, and perhaps most important, we observed that active immunization against MBG was associated with very substantial attenuation of cardiac hypertrophy, cardiac fibrosis and the oxidant stress state. The decreases in cardiac expression of SERCA2a, as well as SERCA enzymatic activity seen with partial nephrectomy, were also markedly attenuated by the active immunization.

Taken together, our data strongly support an important role for MBG in the pathogenesis of experimental uremic cardiomyopathy in the rat. If these data are confirmed clinically,
it is possible that active (or passive) immunization against MBG or, more likely, drug
design based on MBG structure may provide useful clinical strategies against uremic
cardiomyopathy.

Finally, we endeavored to further examine the ROS dependent mechanisms by which
cardiotonic steroids might directly modulate both cardiac function and the physiologic
activity of major calcium handling proteins. While we did not observe significant
changes in the expression of important calcium handling proteins such as SERCA or
PLB, our results demonstrated that ouabain impairs active relaxation in both whole hearts
and isolated myocytes. Ouabain treated hearts and myocytes had increased levels of
carbonylation and tyrosine nitration, decreased SR Ca$^{2+}$ content, lower SERCA2a
activity, and impaired diastolic function. The finding that all of these effects were
reversed with antioxidant therapy lends support to the novel role oxygen radicals may
play in mediating the effects of ouabain. The role of ROS was further supported by the
finding that LV homogenate exposed to H$_2$O$_2$ demonstrated decreased SERCA activity in
a dose dependent fashion similar to the decreases observed with ouabain treatment. The
LC-MS analysis of ouabain treated hearts identified modifications consistent with an
oxidative modification of sulphydryl containing residues. These data suggest that ouabain
impairs SERCA activity in a ROS/RNS dependent manner. Oxidative and nitrosative
modifications of the SERCA protein suggest molecular mechanisms by which ouabain
may alter cardiac Ca$^{2+}$ cycling and physiological function.
CONCLUSIONS

1. In patients with ischemic renal disease undergoing stent therapy, baseline renal insufficiency is associated with higher rates of fatal and non-fatal cardiovascular and renal adverse events. In contrast, improvement in renal function appears to be associated with increased survival.

2. Experimental uremia, induced by 5/6th nephrectomy, causes marked cardiac hypertrophy and changes in cardiac gene expression that could not be explained by only the observed increases in blood pressure.

3. Delayed calcium cycling and impaired contractile function were noted in the myocytes isolated from the hearts of uremic animals. Transcriptional downregulation of the SERCA2a protein appears to account for the impaired diastolic function seen in these myocytes.

4. Experimental uremia induced by partial nephrectomy is associated with virtually all of the molecular and physiological features of clinical uremic cardiomyopathy. Specifically, animals subjected to partial nephrectomy developed systemic oxidant stress along with diastolic dysfunction and cardiac hypertrophy quite consistent with that seen in patients afflicted with chronic renal failure.
5. Infusion of MBG produced almost identical increases in the plasma level of MBG to that seen with partial nephrectomy. These MBG infusions also produced a similar degree of oxidant stress as well as most of the cardiac functional and morphological alterations seen with partial nephrectomy including a significant and substantial amount of cardiac fibrosis.

6. Active immunization against MBG was associated with very substantial attenuation of cardiac hypertrophy, cardiac fibrosis and the oxidant stress state seen in the experimental uremia model.

7. Acute administration of the cardiotonic steroid ouabain modulates SERCA activity in a ROS/RNS dependent manner. Oxidative and nitrosative modifications of the SERCA protein suggest novel molecular mechanisms by which ouabain may alter cardiac Ca\(^{2+}\) cycling and physiological function.
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cardiac contractile function, calcium cycling, and gene expression of proteins


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Biochem Biophys Res Commun 207, 155-159.


ABSTRACT

Patients with chronic renal failure develop a “uremic” cardiomyopathy characterized by diastolic dysfunction, cardiac hypertrophy and systemic oxidant stress. Patients with chronic renal failure also are known to have increases in the circulating concentrations of marinobufagenin (MBG), a cardiotonic steroid. On this background, we investigated a clinical population of patients with ischemic renal disease in order to examine the implications of stabilizing or improving renal function as it related to the effects on cardiac morbidity and mortality. We then employed the 5/6th nephrectomy model (PNX) in the rat in order to examine the molecular mechanisms by which chronic renal failure contributes to cardiac abnormalities and to examine the role of MBG in the systemic oxidant stress state and cardiac changes seen with experimental uremia. Finally, we examined the reactive oxygen/nitrogen (ROS/RNS) dependent mechanisms by which cardiotonic steroids modulate cardiac function.

First, we observed that in patients with renal artery stenosis undergoing stent therapy, baseline renal insufficiency is associated with an increased incidence of morbidity and mortality, independent of other baseline clinical factors. Importantly, improvement in renal function appears to be associated with increased survival. Next, using the PNX model we observed that chronic renal failure leads to alterations in cardiac gene expression and produced alterations in cardiac calcium cycling and contractile function in the rat. Administration of MBG caused comparable increases in plasma MBG, blood pressure, cardiac weight and diastolic dysfunction as PNX at 4 wk. Decreases in the
expression of the cardiac sarcoplasmic reticulum ATPase (SERCA2a), cardiac fibrosis and systemic oxidant stress were observed with both MBG infusion and PNX, while active immunization against MBG attenuated these changes without affecting blood pressure. Finally, acute administration of the cardiotonic steroid ouabain modulated SERCA activity in a ROS/RNS dependent manner. Proteomic analyses by immunoblotting, immunoprecipitation, and liquid chromatography/mass spectrometric of left ventricles revealed oxidative and nitrosative modifications of the SERCA protein, suggesting a molecular mechanism by which ouabain modulates cardiac function.

Taken together, these data suggest that the increased concentrations of MBG are important in the cardiac disease and oxidant stress state seen with renal failure.