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Interactions Between Aldosterone, Spironolactone and the Cardiotonic Steroids

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INTRODUCTION

We have demonstrated that both partial nephrectomy (PNx) and marinobufagenin (MBG) administration induce cardiac fibrosis in the rat (Kennedy et al., 2006) and that active immunization against MBG attenuates this process. Elevated levels of Aldosterone also have been implicated in the progression of cardiac fibrosis in this model Green et al., 1996) as well as other settings. Interestingly, the in vivo studies which implicate Aldosterone in cardiac fibrosis require concurrent volume expansion (Young and Funder 2002), and these settings are associated with increased MBG (Fedorova et al., 1998). To address cross-talk between Aldosterone and MBG, we first studied PNx rats which were adrenalectomized (PNx+ADx), but supplemented with physiological amounts of Aldosterone(A) and dexamethasone (D) for 4 wk. PNX alone induced marked increase in SBP, heart/body weight ratio (HBR) and tissue fibrosis as we have previously reported (all P<0.01) (Elkareh et al., 2007), while PNx+ADx rats demonstrated decreased heart weight/body weight ratios and fibrosis versus PNx, but without changes of SBP (all p[0.01). Interestingly, PNx+ADx had a marked reduction in plasma MBG compared to PNx (Elkareh et al., 2007) (p<0.05). In the present study we investigated the chronic infusion of spironolactone on the cardiac fibrosis and cardiac dysfunction caused by chronic renal failure in rat model.
LITERATURE

Chronic Kidney Disease Epidemiology

Chronic kidney disease (CKD) is a worldwide public health problem. There is a rising incidence and prevalence of kidney failure in the United States (K/DOQI clinical practice guidelines, 2002). The total cost of the ESRD program in the US was approximately $27 billion in 2003 (Grapso and Oreopoulos 1996). Chronic renal failure is a slowly progressive loss of renal function over a period of months or years and defined as an abnormally low glomerular filtration rate. Patients with CKD is currently complicated by high prevalence of cardiomyopathy arteriosclerosis, which is characterized by marked left ventricular hypertrophy, cardiac hypertrophy, and diastolic dysfunction, is still poorly understood (Sarnak et al., 2003; Baker et al., 1969).

Hypertension, extracellular volume expansion and anemia have been implicated as contributing factor to the cardiac disease seen in those patients (Elkareh et al., 2007). Patients with renal failure usually develop cardiac complications. In chronic renal disease (CRD) patients treated with hemodialysis in the US, mortality rates exceed 20% per year with more than 50% attributed to cardiac mortality (United States Renal Data System, 1997), recent studies suggest that the most common form of heart disease in patients is characterized by diastolic dysfunction and left ventricular hypertrophy (Parfrey et al., 1991). Interestingly, even mild degrees of renal failure cause a significant increase in cardiovascular disease (Kennedy et al., 2006).
Cardiotonic Steroids Physiology and Action

Cardiotonic steroids, which are also called digitalis-like steroid hormones are a family of steroid hormones synthesized in and released from the adrenal gland, which bind to and inhibit the activity of the cell surface enzyme $\text{Na}^+/\text{K}^+$ ATPase, which suggest its effects and physiological role on regulation of ion transport by $\text{Na}^+/\text{K}^+$ ATPase. The binding of cardiotonic steroids to $\text{Na}^+/\text{K}^+$ ATPase leads to a change in cytosolic $\text{Na}^+$ and, indirectly, in cytosolic $\text{Ca}^{2+}$ concentrations, changing the $\text{Ca}^{2+}$ content of the sarcoplasmic reticulum and $\text{Ca}^{2+}$ signaling and leads to the positive ionotropic effect. This binding also activates signal transduction cascades, which will stimulate endocytosis and affect cell proliferation and growth (Nesher et al., 2007; Schoner 2002).

In previous studies, it has been observed that steroid molecules like ouabain, MBG and digoxin, which bind to the plasmalemmal Na/K-ATPase, accumulate in patients of chronic renal failure. These molecules have been referred to as cardiotonic steroids. Considerable effort was put into the measurement of these molecules and their role in renal and cardiac renal physiology (Wollenweber et al., 1968).

Our recent work has established that the cardiotonic steroid’s, MBG signaling through the $\text{Na}^+/\text{K}^+$ ATPase induces natriuresis and uremic cardiomyopathy in PNx rats and in MBG infusion mini-pump rats and subsequently increases blood pressure, cardiac hypertrophy, and fibrosis in those rats (Kennedy et al., 2006; Elkareh et al., 2007; Johansson et al., 1999).

Studies from the laboratory of Dr. Bagrov at the NIH have detected MBG and bufalin in body fluids by using antibody-based assays (Bagrov et al., 1998). Komiyama
and colleagues were able to detect MBG and a derivative, telocinobufagin, in human plasma of ESRD patients (Komiyama et al., 2005).

Elevated levels of circulating MBG have been demonstrated in clinical and in experimental renal disease at 4 wk of experimental renal failure (Elkareh et al., 2007; Kennedy et al., 2006; Dorros et al., 2002). The MBG was found to be a regulator of renal sodium excretion and a hypertension-promoting factor; plasma levels of MBG were elevated after plasma volume expansion, and also elevated in pregnant rats model of pre-eclampsia, antibody to MBG lowers the blood pressure in these model Fedorova et al., 1998; 2002; 2005).

Marinobufagenin has a greater affinity for the alpha-1 subunit of \( \text{Na}^+/\text{K}^+ \) ATPase, which is resistant to oubain (Fedorova and Bagrov 1997; Fedorova et al., 2000; Schoner 2002). Cardiotonic steroids induce signaling pathway through the plasmalemmal \( \text{Na}^+/\text{K}^+ \) ATPase and cause an increase in production of oxygen reactive species (ROS). The interaction between ROS and other circulating pump inhibitors can cause a significant modulation of transcription factors that result in the activation of multiple pathways of cardiac hypertrophy (Kennedy et al., 2006). Recently, our group has shown that administration of antibodies raised against MBG in Sprague Dawley rats given a high salt diet reduced \( \text{Na}^+/\text{K}^+ \) ATPase endocytosis and sodium excretion (Periyasamy et al., 2005).

Marinobufagenin, whose concentration is increased after myocardial infarction, may show natriuretic properties because it inhibits the alpha-1 isoform of \( \text{Na}^+/\text{K}^+ \)-ATPase more than other sodium pump isoforms (Schoner 2002). The increased production of MBG (endogenous digitalis-like compounds) may also contribute to hypertension by
inhibition of Na⁺/K⁺-ATPase in cardiovascular tissues (Schoner 2002). The development of congestive heart failure is correlated with down-regulation of the alpha-1 isoform of the Na⁺/K⁺ ATPase in left ventricular myocardium (Manunta and Ferrandi 2004).

**Aldosterone Origin, Mechanism of Action and the Regulating Factor**

An aldosterone is a hormone, which is produced by adrenal gland in the zona glomerulosa, the final steps in the conversion of corticosterone to aldosterone, by addition of an hydroxyl group at the 18-carbon position and then oxidation to an aldehyde, which occur only in the zona glomerulosa (White et al., 1987; White 1994).

Aldosterone acts primarily in the distal nephron of the kidney to increase the reabsorption of Na⁺ and Cl⁻ and the secretion of K⁺ and H⁺, aldosterone acts by diffusing into the tubular cell and then attaching to a cytosolic receptor (Bastl and Hayslett 1992; Harisberger and Rossier 1992). Then the hormone-receptor complex migrates to the nucleus, where it enhances messenger RNA and ribosomal RNA transcription. This in turn is translated into new proteins called aldosterone-induced proteins (AIPs) (Minuth et al., 1988; Verrey 1999).

The aldosterone-induced elevation Na⁺ in luminal; this Na⁺ is then returned to the systemic circulation by the Na⁺-K⁺-ATPase pump (Bastl and Hayslett 1992). The movement of Na⁺ through its channel creates a lumen-negative potential difference. Electroneutrality is maintained in this setting either by K⁺ secretion from the cell into the lumen or by passive Cl⁻ reabsorption via the paracellular pathway (Bastl and Hayslett 1992).
The increase in activity Na+-K+-ATPase is probably induced in part by aldosterone as aldosterone-induced proteins synthesis may be subunits of the Na+-K+-ATPase pump (Bastle and Hayslett 1992; Fujji et al., 1990).

The rennin-angiotensin-aldosterone system has been implicated in the pathophysiology of heart failure, its effect is attributed to the activation of mineralocorticoids receptor, which will cause salt and water retention and consequence will lead to increase afterload (Jennings et al., 2005).

Aldosterone secretion may be increased in hyponatremic patient who is volume-depleted and reduced in hypernatremic patient but may be reduced in a patient who is volume-expanded (Taylor et al., 1987; Merrill et al., 1989; Barber et al., 1956). Aldosterone has been implicated of cardiovascular injury in various diseases. In one study, treatment of male 22 wk-old spontaneously hypertensive rats with mineralocorticoid antagonism prevents the adverse cardiac effects of hypertension, hydroxyproline concentration in the right and left ventricle was decreased and reduced in myocardial fibrosis (Susic et al., 2007). The mechanisms that aldosterone promotes and causes myocardial fibrosis by upregulating of angiotensin II receptors, alternation of plasminogen activator inhibitor-1, generation of ROS, and activation of transforming growth factor TGF-β (Jennings et al., 2005). The elevation of ROS and TGF-β play a major role in progression of cardiac remodeling via activation of matrix metalloproteinases, and consequence will stimulate collagen turnover, which will cause cardiac hypertrophy and fibrosis (Cucoranu et al., 2005).
There is evidence that increasing aldosterone level has an impact on kidney, heart and vasculature by promoting vascular remodeling, and collagen formation (Epstein 2006). Aldosterone may mediate and play a role in the pathogenesis of myocardial injury by activation of vascular inflammation and subsequent myocardial fibrosis and increased peripheral arterial resistance (Zannad and Radauceanu 2005).

In patients and in rat models of myocardial infarction, a common complication is the presence of excessive collagen deposition that causes progressive heart disease. Several hormones, including aldosterone and pro-fibrotic cytokines, control this process. Procollagen processing by procollagen C-proteinase(s) is augmented by procollagen C-proteinase enhancer proteins, which is increased by aldosterone in addition to stimulation of collagen-I expression in cultured heart fibroblasts. Treatment with aldosterone blockade reduced this stimulatory effect by approximately 50% (Kessler-Icekson et al., 2006).

**Spironolactone Action and Benefit**

Spironolactone competitively inhibits the aldosterone dependent sodium-potassium exchange site in the distal convoluted renal tubule (Kleyman and Cragoe 1988). In patients treated with diuretics, spironolactone may also act in the distal tubule, diminishing the number of Na\(^+\)-Cl\(^-\) cotransporters (Abdallah et al., 2001).

Spironolactone and valsartan, an angiotensin blocker, prevent cardiac and perivascular fibrosis and suppress the cellular hypertrophy of myocytes (Okada et al., 2006). Major clinical trials involving spironolactone and eplerenone have shown
significant outcome in the treatment of congestive heart failure (CHF). The New York Heart Association reported that patients of class III and class IV CHF treated with spironolactone had a 30% relative risk decrease in mortality caused by cardiovascular diseases (Jewell et al., 2006). Spironolactone has been shown to increase endothelial nitric oxide bioavailability, which will improve endothelial function (Dawson et al., 2004). Aldosterone blockade by spironolactone has been shown to be effective in reducing cardiovascular mortality rates in patients with systolic left ventricular dysfunction due to ischemic or non-ischemic causes and also effective in left ventricular hypertrophy patients with essential hypertension (Pitt 2004). Several articles have demonstrated that spironolactone may overcome the effects of aldosterone in inducing cardiac fibrosis and may also antagonize both renal and extra-renal effects (Zannad and Radauceanu 2005). The mechanism by which spironolactone decreases fibrosis is by reducing the circulating level of the procollagen type III N-terminus, which is indicative of collagen turnover (Jennings et al., 2005).
MATERIAL AND METHOD

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 University of Toledo, Medical Health Campus, Institutional Animal Use and Care (IACUC) Committee.

Male Sprague Dawley rats (250-275 grams) were divided into groups. One group was subjected to 5/6th nephrectomy and a second group was subjected to sham operation as described in a recent report from our laboratory (Kennedy et al., 2006), sham surgery was performed with placement of a minipump placed (model 2004, Alzet, Palo Alto, CA) subcutaneously making flank incisions and infusing spironolactone (Sigma) at 20 mg/kg/d in the third group, and partial nephrectomy (PNx) with minipumps infusing spironolactone at 20 mg/kg/d in the final group. The most commonly used dose of spironolactone used in other studies is 20 mg/kg/d (Kennedy et al., 2006; Feria et al., 2003).

In addition to these maneuvers, a group of PNx animals was subjected to adrenalectomy procedure (PNx+ADx). For sake of long term survival, adrenalectomized animals were given mineralocorticoids and glucocorticoids through mini-pumps (model 2004, Alzet, Palo Alto, CA) placed subcutaneously to maintain electrolyte balance of each rat in order to deliver 2.0 μg/100 g BW/d of aldosterone (Sigma A6628) and 1.2 μg/100 g BW/d of dexamethasone (Sigma D1756) (Elkareh et al., 2007). After surgery, the rats were allowed to recover for 4 wk with easy access to chow mix food (Rodent
Laboratory Chow 5001, PMI Nutrition International, Inc., Brentwood, MO) and water. After 4 wk, the animals were euthanized by administering (50 mg/kg) of Nembutal intraperitoneally.

Systolic blood pressure was measured in conscious animals by using the tail-cuff method and equipment made by IITC, Inc. (Amplifier model 229, Monitor model 31, Test chamber Model 306; IITC Life Science. Blood pressure was measured once weekly for 4 wk, as previously described (Kennedy et al., 2006).

**Left Ventricular Hemodynamics**

Prior to sacrifice the rats, left ventricular pressure and volume were determined by placement of a 2F Millar Microtip pressure-volume catheter (SPR-838; Millar Instruments, Inc., Houston, TX) into the left ventricle through a carotid artery insertion as previously described (Kennedy et al., 2006). After stabilization for 10-15 min, the cardiac measurements which will allow us to further characterize our heart failure models in exquisite detail. Specifically, real time pressure-volume loop measurements were obtained to characterize the function and performance of the heart throughout all phases of the cardiac cycle, the signals were continuously recorded for 15-20 min, stored, and displayed on a computer (Elkareh et al., 2007; Kennedy et al., 2006).

The end-diastolic volume, end-systolic volume, ejection fraction and relaxation time constant (tau, $\tau$) of isovolumic relaxation were computed using a cardiac pressure-volume analysis program (PVAN3.5; Millar Instruments, Inc.) as previously described (Elkareh et al., 2007; Kennedy et al., 2006).
After measuring the intraventricular pressure, blood was collected from each animal; then, organs were removed and the heart weight was normalized to body weight (HW/BW).

**Measurement of MBG and Ouabain-Like Compound**

The MBG and ouabain-like compound (OLC) were determined from plasma by using an enzyme-linked immunosorbent assay (ELISA) technique as described previously (Kennedy et al., 2003).

**Isolation of Cardiac Fibroblasts**

Isolation of cardiac fibroblasts was carried out as previously described by Brilla et al. (1994) with modifications. Briefly, male Sprague Dawley rats weighing 250-300 grams were used to obtain fibroblast from the hearts, the rats were anesthetized with (50 mg/kg) of Nembutal intra-peritoneally and heparanized (2000 unit/kg). The hearts were removed and perfused under sterile condition via the ascending aorta with Joklik's medium (Sigma-Aldrich, St. Louis, MO) in a modified Langendorff apparatus. After 5 min of perfusion, the perfusate was placed in Joklik's medium containing 0.1% collagenase type 2 (Worthington Biochemical, Lakewood, NJ) and 0.1% BSA which was circulated for 15-25 min until the heart became flaccid. Ventricles were excised and finely cut, and shaken in Joklik's modified medium with 0.1% collagenase and 0.1%BSA for15 min. Cells /tissue suspension was allowed to settle for 15 min and was centrifugated at 500 rpm for 10 min. The supernatant then was centrifugated at 1500 rpm for 15 min. The resulting pellet was suspended in DMEM supplemented with antibiotics
(penicillin/streptomycin/fungizone) plus 15% FBS (Hyclone, Logan, UT), and plated in a 10-cm plate and incubated at 37°C Celsius under 5% carbon dioxide concentrations (Elkareh et al., 2007; Brilla et al., 1994).

**Western Blot Analysis**

Western Blot analysis was performed on proteins isolated from cell lysates or from tissue homogenates. Briefly, for tissue analysis, the left ventricles from the heart were homogenized in ice-cold buffer (pH 7.0) containing 25mM imidazole and protease inhibitors. The homogenate was centrifuged at 12,000 G for 10 min at 4°C Celsius. The protein was quantified in the supernatant and the proteins were solubilized in sample buffer (2% SDS, 5% β-mercaptoethanol, 20% glycerol, 0.005% bromophenol blue and 50mM Tris-HCl pH 7.0). The proteins were resolved on an SDS-PAGE using 10% or 4-15% gel as previously described (Kennedy et al., 2003). The proteins from the gel were electro-transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in 20 mM Tris-HCl (pH 7.5, 150 mM NaCl, and 0.1% Tween 20). Goat anti-type 1 collagen antibody (Southern Biotech, Birmingham, AL) was used to probe for procollagen. The antigen-antibody complex was detected using donkey anti-goat antibody coupled to horseradish peroxidase (Santa Cruz, CA) and using ECL and ECL-plus purchased from Amersham Biosciences (Piscataway, NJ), Anti-ERK-1 polyclonal antibody, anti-phospho-ERK-1 mAb, and anti-rabbit antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).
All the membranes were simultaneously blotted with either anti-actin or anti-tubulin antibodies (mouse monoclonal, Santa Cruz) (Kennedy et al., 2003). For the cell lysates, the cells were grown to confluence and starved for 18 h in DMEM with 1% FBS. The cells then were treated with MBG or spironolactone for 24 h. The cells were washed with phosphate buffered saline (BPS) twice and exposed to lysis buffer. The proteins from the cell lysate were fractionated and detected as described above (Elkareh et al., 2007).

**Histology and Immunohistochemistry**

Left ventricle sections were immediately fixed in 4% formalin buffer solution (pH 7.2) for 18 h, dehydrated in 70% ethanol, and then embedded in paraffin and cut with a microtome. For the purposes of measuring fibrosis in myocyte cross-sectional areas, trichrome staining was performed as we have previously reported (Elkareh et al., 2007; Kennedy et al., 2006).

Myocyte cross-sectional areas were measured on samples derived from eight animals in each experimental group using standard planimetric methods (Baker et al., 1969; United States Renal Data System 1997) with ImageJ software (ImageJ 1.36b, National Institutes of Health, USA). On other sections, immuno-staining was performed using anti-collagen 1 (ab6308, 1:50 dilution, Abcam Inc, Cambridge, MA) and anti-smooth muscle actin (anti-αSMA, clone 1A4, Sigma-Aldrich). For quantitative morphometric analysis of fibrosis, five random sections of trichrome slides were electronically scanned which were subsequently analyzed using Image J (version 1.32j)
software (National Institutes of Health, USA http://rsb.info.nih.gov/ij/). The scoring of fibrosis estimated by the RGB images with a macro written by the authors (JIS), as previously described (Kennedy et al., 2006). For confirmation of immunohistochemical findings, quantitative protein determination on left ventricular homogenates was performed using Western blot as described above (Elkareh et al., 2007; Kennedy et al., 2006).

**Collagen Synthesis**

3H-Proline incorporation study by cardiac fibroblasts was done to investigate the rate of collagen synthesis. Cardiac fibroblasts were isolated from Wistar rats and the cells were grown to confluence in DMEM with 15% FBS. The cells then were incubated in DMEM supplemented with 1% FBS for 18 h before treatment, then the cells were treated for 24 h in 1% FBS fresh medium. 3H-proline (1μCi/ml) was added 12 h prior to the termination of the treatment. The experiments were terminated by washing the cells twice with PBS and twice with ice-cold trichloroacetic acid (10%). The precipitate was solubilized in 100 μL of 0.5 N NaOH and 0.1% SDS then aliquoted from each well with 5 mL scintillation fluid were later counted in a liquid scintillation counter (Elkareh et al., 2007; Brilla et al., 1994).

**Statistical Analysis**

Data presented are mean ± SEM. Significance of the differences among groups were tested ANOVA. All comparisons passed the normality test. Statistical significance was defined when p value was <0.05.
RESULTS

Effect of Experimental Renal Failure and Spironolactone on Blood Pressure and Hemodynamics

We have previously reported that rats with PNX developed increases in blood pressure as well as increases in plasma concentration of MBG, heart size, and diastolic dysfunction (Elkareh et al., 2007). In our current studies, we showed that PNX compared with sham operated control rats after 4 wk of surgery had higher SBP (186 ± 26.9 versus 107 ± 5.9), We also observed that PNx rats treated with spironolactone had higher SBP than controls (130 ± 6.7 versus 107 ± 5.9), but less than PNX alone. On the other hand, there are no significant changes in SBP in the rats treated with spironolactone compared to the controls (99 ± 3.1 versus 107 ± 5.9), (Figure 1).

Effect of Experimental Renal Failure and Spironolactone on Cardiac Morphology and Biochemistry

Rats subjected for partial nephrectomy (PNX) had marked increases in the ratio of heart weight/body weight compared to control animals and spironolactone treated animals; the ratio of heart weight/body weight in PNX, PNX and spironolactone, spironolactone alone, compared to controls were 0.33 ± 0.03, 0.28 ± 0.01, 0.28 ± 0.004, 0.27 ± 0.0016, respectively) (Figure 2).
Figure 1: Representative Systolic blood pressure 4 wk after sham operation (SS, n=8) sham surgery with spironolactone infusion (20 mg/kg/d) (SSP, n=8), partial nephrectomy (PS, n=8), partial nephrectomy with spironolactone infusion (20 mg/kg/d. (PS-SP, n=7). $P<0.05$ versus Sham, $#P<0.05$ versus PNx, $##P<0.01$ versus PNx.
Figure 2: Represents Heart weight/body weight (HW/BW) ratio 4 wk after sham operation (SS, n=8) sham surgery with spironolactone infusion (20 mg/kg/d) (SSP, n=7) partial nephrectomy (PNx, n = 8), partial nephrectomy with spironolactone infusion (20 mg/kg/d (PS-SP, n=7). $P<0.05$ versus Sham, $\#P<0.05$ versus PNx, $\##P<0.01$ versus PNx.

Western Blot analysis of left ventricular tissues manifested increases in procollagen expression in partially nephrectomized (PNx) animals. Treatment of PNX animals with spironolactone prevented or attenuated the increases in procollagen expression is shown in (Figure 3).
Figure 3: Gels were loaded with 100-µg left ventricle homogenate protein. Representative procollagen expression blots shown in the left ventricular cardiac homogenate 4 wk after Sham (SS, n=7) sham surgery with spironolactone infusion (20 mg/kg/d) SSP, n=5), partial nephrectomy (PNx, n=6), partial nephrectomy with spironolactone infusion(20 mg/kg/d. PS-SP, n=6). P<0.05 versus Sham, #P<0.05 versus PNx, ##P<0.01 versus PNx.

Partial nephrectomy is also associated with activation of ERK. Treatment of PNX animals with spironolactone attenuated activation of ERK is shown in Figure 4.
Figure 4: Gels were loaded with 10-µg left ventricle homogenate protein. Representative activation of phospho-ERK and total ERK blots shown in the left ventricular cardiac homogenate 4 wk after Sham (SS, n=6) sham surgery with spironolactone infusion (SSP, n =5), partial nephrectomy (PNx, n=5), partial nephrectomy with spironolactone infusion. (PS-SP, n=6). $P<0.05$ versus Sham, $#P<0.05$ versus PNx, $##P<0.01$ versus PNx.

Partial nephrectomy resulted in marked increases in cardiac fibrosis as assessed by quantitative morphometric fibrosis scoring for trichrome slides of left ventricular
cardiac sections after 4 wk compared to control. Treatment of partial nephrectomy rats with spironolactone attenuated the histological changes seen in animals of partial nephrectomy, (Figure 5a and 5b).

Figure 5. Left Ventricular Trichome
Figure 5: Panel (a): Representative Masson’s trichrome sections of left ventricular cardiac tissue 4 wk after Sham (SS, n=5) sham surgery with spironolactone infusion (SSP, n=5), partial nephrectomy (PNx, n=5), partial nephrectomy with spironolactone infusion (PS-SP, n=6). \( P<0.05 \) versus Sham, \( \#P<0.05 \) versus PNx, \( \##P<0.01 \) versus PNx. Panel (b) Represents the ratio of collagen in each group.

Also, the myocyte cross-sectional area in PNx-Spironolactone was smaller than that with PNx alone (Figure 6).
Figure 6: Ventricular cross sectional areas determined by trichrome stains of tissue obtained from sham, Spironolactone, Pnx, and PNx + spironolactone animals (each group: n=5 animals, around 100 measurements averaged to determine mean for each animal.

**Effect of MBG and Spironolactone on the Cell Culture of Primary Cardiac Fibroblast**

In primary culture of rat fibroblasts grown to confluence, the effects of MBG and spironolactone on procollagen content of cultured cardiac fibroblasts were examined and detected by Western blotting. Treatment of primary cultured adult fibroblasts with 10 nM MBG for 24 h increased the accumulation of procollagen versus control; while a decrease in procollagen content was noted after treatment with 0.1 µM spironolactone. On the
other hand, adding spironolactone to MBG attenuated the increase of procollagen expression (Figure 7a).

Figure 7: Procollagen Expression

Figure 7: Represent (a) Western blot for procollagen and quantitative densitometric data in response to MBG 10 nM, Spironolactone 100nM (all n=6), (b), relative proline incorporation in matrix in response to MBG 10 nM, Spironolactone (100 nM, 1µM), and both doses of Spironolactone with 10 nM MBG, (All n=10).
We also observed that when physiological concentrations of MBG (10nM) were added to the culture media, the rate of incorporation of proline into collagen over 24 h markedly increased. Interestingly, while the effects of MBG on proline incorporation was attenuated by co-incubation with spironolactone (1µM, 0.1 µM) (p<0.01), the effects of (1µM, 0.1 µM) neither stimulated proline incorporation by itself nor in an additive fashion with MBG (Figure 7 b).
Effects of Partial Nephrectomy and Adrenalectomy on Cardiac Morphology, Blood Pressure, Hemodynamics, and Biochemistry

To further examine whether increased concentrations of MBG were required for the changes in cardiac phenotype following PNx, a separate group of animals (n=11) also were subjected to simultaneous adrenalectomy and placement of an osmotic minipump to provide physiological replacement of dexamethasone and aldosterone (PNx-ADx). These animals developed a similar degree of hypertension (SBP = 193 ± 6 mmHg determined with tail cuff, p=NS compared with PNx) but were noted to have much lower plasma concentrations of MBG (325 ± 65, p<0.01) and plasma concentration of aldosterone (228±65, p<0.01) as well as substantially lower heart/weight body weight ratio (3.30 + 0.10 g/kg body weight, p<0.05) compared with PNx alone. Interestingly, the decreases in end-systolic (56 + 5, p< 0.05) and end-diastolic (185 + 14, p=NS) volumes as well as the increases in τ value (12.1+ 0.5, p< 0.05) and the EDPVR (0.038 + 0.004mmHg/μl) seen with PNx were attenuated by instantaneous adrenalectomy. Moreover, the animals subjected to PNx-ADx had almost no evidence of cardiac fibrosis based on trichrome staining (Figure 8a) or immunohistochemistry staining for collagen-1 or α smooth muscle actin (Figure 8b) (Elkareh et al., 2007).
Figure 8. Trichrome Staining

Trichrome

PNx

PNx+ADx
Figure 8: (a) Representative trichrome stained microscopic sections from rats subjected to partial nephrectomy (PNx, n=8) and partial nephrectomy and coincident adrenalectomy (PNx-ADx, n=11). (b) immunohistochemistry staining for collagen-1 or α smooth muscle actin.

Western Blot analysis of left ventricular tissues was associated with increases of procollagen expression in partial nephrectomy (PNx) animals, (Figure 9).
Figure 9: Gels were loaded with 75-µg left ventricle homogenate protein. Representative procollagen expression blots shown in the left ventricular cardiac homogenate 4 wk after sham operation (SS, n=6) and subjected to partial nephrectomy (PNx, n=7) and partial nephrectomy and instantaneous adrenalectomy (PNx-ADx, n=8). *P<0.05 versus Sham, #P<0.05 versus PNx, ##P<0.01 versus PNx.

Partial nephrectomy is associated with the activation of phospho-ERK, while PNx + adrenalectomy rats with physiologic replacement of aldosterone and dexamethasone...
prevented or attenuated the increases in procollagen expression and activation of ERK (Figure 10) (Elkareh et al., 2007).

Figure 10. Procollagen Expression and Activation of ERK

Figure 10: Gels were loaded with 10-µg left ventricle homogenate protein. Representative activation of phospho-ERK and total ERK blots shown in the left ventricular cardiac homogenate expression blots shown in the left ventricular cardiac homogenate 4 wk after sham operation (SS, n=6) and subjected to partial nephrectomy...
(PNx, n=7) and partial nephrectomy and instantaneous adrenalectomy (PNx-ADx, n=8). $P<0.05$ versus Sham, $P<0.05$ versus PNx, $P<0.01$ versus PNx.
DISCUSSION

Cardiac diseases are directly responsible for the high morbidity and mortality rates seen in patients with chronic renal failure; patients with end-stage renal disease develop cardiac disease with exceptional frequency (Hakim et al., 1996). Such renal diseases coupled with cardiac dysfunction are denoted cardiomyopathies. At the same time, patients with chronic renal failure develop cardiac hypertrophy and diastolic dysfunction (Kennedy et al., 2006). Interestingly, patients of cardiomyopathies show increased cardiac fibrosis in most cases (Elkareh et al., 2007).

Our group has noticed that Cardiotonic steroids including (MBG) induce signal transduction through Na⁺/K⁺-ATPase, which consequence in activation of EGFR, Scr, production of ROS, and finally activation of p42/44 mitogen-activated protein kinases, which are considered as a mediators of cardiac hypertrophy (Elkareh et al., 2007; Xie and Askari 2002).

In addition, we have previously proved that rats subjected to partial nephrectomy (PNx), as well as rats equipped with MBG mini-pump developed cardiac fibrosis and cardiac hypertrophy along with alterations of diastolic function, elevation of SBP, and the marked elevation of aldosterone level. The results are quite consistent with what we have seen in patients afflicted with chronic renal failure (Himmelfarb et al., 2002; Elkareh et al., 2007). On the other hand, such dysfunctions were attenuated in rats immunized against MBG and later subjected to partial nephrectomy (Elkareh et al., 2007).

In the current study we showed that rats subjected to partial nephrectomy (PNx) showed an increase in heart size, blood pressure, and systolic function as well as impaired
diastolic function. Next, we noted that spironolactone administration to rats subjected to partial nephrectomy (PNx) attenuated these phenotypical changes including attenuation of cardiac hypertrophy, cardiac fibrosis and depreciation of SBP. Based on results of Western Blot analysis of cardiac ventricular tissue, we also observed a decrease in the expression of both procollagen and phospho-ERK in partially nephrectomized rats treated with spironolactone rats versus rats subjected to partial nephrectomy alone. Treatment also prevented MAPK activation with PNx. To investigate the mechanism by which this occurred, we studied isolated primary cardiac fibroblasts grown to confluence. We observed that administration of physiological concentrations of MBG in directly stimulated procollagen expression in the fibroblasts to produce more collagen. This increase in collagen production also was observed with other cardiotonic steroids (Elkareh et al., 2007), adding of physiological concentrations of spironolactone to fibroblast deceased procollagen expression, from the other side, this effects of MBG was blocked co-incubation with spironolactone. We also observed that the increases in collagen production by adding MBG were associated with increases in proline incorporation attenuated by adding spironolactone.

These data suggest that spironolactone antagonize the effect of MBG causing fibrosis. Bricker, DeWardener and others specifically demonstrated that an inhibitor of the plasmalemmal Na⁺/K⁺ ATPase would accumulate in the serum and cause organ dysfunction (deWardener 1996).

In our previous study we point out that experiments in the PNx-ADx model were performed because we reasoned that since adrenal cells grown in culture appear to make
MBG (Dmitrieva et al., 2000; Fedorova et al., 2005). However, while our data in the PNx-ADx animals sustain the concept that the adrenal gland is the major site of MBG production in vivo, but not the only site (Elkareh et al., 2007). More work will be necessary to explain exactly where MBG is produced under normal and pathological circumstances.
CONCLUSIONS

Marinobufagenin appears to produce fibrosis \textit{in vivo} as well as stimulate cultured fibroblasts to produce increased amounts of collagen. This stimulation of collagen production appears to involve elements of plasmalemmal Na$^+$/K$^+$ ATPase signaling and can be blocked by spironolactone. We propose that some of spironolactone’s clinical effects may be due to inhibition of MBG induced fibrosis.
SUMMARY

Cardiotonic steroids (MBG), signaling through the Na⁺/K⁺ ATPase induces uremic cardiomyopathy in PNx rats causing cardiac hypertrophy and fibrosis. Spironolactone protects against cardiac hypertrophy and fibrosis by preventing signaling through the Na/K-ATPase in vivo, as evidenced by attenuation of MAPK and Src activation. Spironolactone also prevents MBG induced procollagen expression in cardiac fibroblasts.


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ABSTRACT

We have reported that rats subjected to partial nephrectomy (PNx) or administration of marinobufagenin (MBG) developed an increase in blood pressure, heart size, diastolic dysfunction, and cardiac fibrosis. Spironolactone (S) has been demonstrated to attenuate cardiac fibrosis in both experimental animals and humans with heart failure. We investigated the effects of treatment of S in the cardiomyopathy. Rats were divided into four groups: one group PNx and a second group sham surgery (C), placement of a minipump of S in another group and PNx + S in the final group. Rats were studied at 4 wk. PNx rats had higher systolic blood pressure, cardiac fibrosis than C. S did not differ significantly from C. PNx + S had substantially attenuated cardiac growth and fibrosis and decreased blood pressure (all p<0.01). These data suggest that S antagonizes MBG induced signaling which leads to cardiac fibrosis in the PNx model and raises the tantalizing question whether this mechanism explains at least part of the clinical beneficial effects of S on cardiac fibrosis in this and other settings.