

Spironolactone Attenuates Experimental Uremic Cardiomyopathy by Antagonizing Marinobufagenin

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Abstract—Spironolactone has been noted to attenuate cardiac fibrosis. We have observed that the cardiotonic steroid marinobufagenin plays an important role in the diastolic dysfunction and cardiac fibrosis seen with experimental renal failure. We performed the following studies to determine whether and how spironolactone might ameliorate these changes. First, we studied rats subjected to partial nephrectomy or administration of exogenous marinobufagenin. We found that spironolactone (20 mg/kg per day) attenuated the diastolic dysfunction as assessed by ventricular pressure-volume loops and essentially eliminated cardiac fibrosis as assessed by trichrome staining and Western blot. Next, we examined the effects of spironolactone and its major metabolite, canrenone (both 100 nM), on marinobufagenin stimulation of rat cardiac fibroblasts. Both spironolactone and canrenone prevented the stimulation of collagen production by 1 nM marinobufagenin but not 100 nM marinobufagenin, as assessed by proline incorporation and procollagen 1 expression, as well as signaling through the sodium-potassium-ATPase, as evidenced by protein kinase C isoform δ translocation and extracellular signal regulated kinase 1/2 activation. Both spironolactone and canrenone also altered ouabain binding to cultured porcine cells in a manner consistent with competitive inhibition. Our data suggest that some of the antifibrotic effects of spironolactone may be attributed to antagonism of marinobufagenin signaling through the sodium-potassium-ATPase. (*Hypertension*. 2009;54:1313-1320.)

Key Words: cardiomyopathy ■ renal failure ■ cardiotonic steroids ■ collagen ■ fibrosis

Cardiac fibrosis oftentimes complicates congestive cardiomyopathies, and it has been suggested that aldosterone may directly cause cardiac fibrosis.¹ Spironolactone is a synthetic steroid molecule that has been characterized as a mineralocorticoid receptor antagonist.^{2,3} In recent years, it has been found that spironolactone can reduce the artery stiffness and left ventricular mass index in cardiomyopathic conditions.⁴⁻⁶ Mechanistically, spironolactone treatment inhibits angiotensin II and aldosterone-induced activation of epidermal growth factor receptor/extracellular signal regulated kinase (ERK), NAD(P)H oxidase/lectin-like oxidized low-density lipoprotein receptor 1, and ρ -kinase pathways.⁷

In addition to a well-defined ability to prevent the binding of aldosterone to the mineralocorticoid receptor, spironolactone and its major metabolite, canrenone,^{8,9} also interact with the plasmalemmal sodium-potassium-ATPase (Na/K-ATPase).¹⁰ Both have been shown to competitively inhibit ouabain and digoxin binding, and the latter has even been used clinically with the aim of combating digitalis toxicity.¹¹ Depending on the experimental conditions, spironolactone or canrenone has been characterized as either a pure competitive antagonist of

ouabain or a “partial inhibitor” of Na/K-ATPase enzymatic (ion pumping) function. We have observed previously that spironolactone functions as an inotrope in vitro.¹² Moreover, this occurs at pharmacologically relevant concentrations of spironolactone and, in isolated cardiac myocytes, spironolactone stimulation causes very similar changes in calcium cycling to that seen with digoxin or ouabain.¹³

On this background, our laboratory has observed that the cardiotonic steroid marinobufagenin (MBG) is responsible for many of the clinical features of experimental uremic cardiomyopathy, including cardiac fibrosis.¹⁴⁻¹⁶ Interestingly, although the model of uremic cardiomyopathy that we use, specifically the fifth/sixth nephrectomy, is associated with marked increases in circulating aldosterone concentrations, antagonism by active immunization with an MBG-albumin conjugate, a process that produces a rather specific antibody response against MBG with negligible cross-reaction to aldosterone, ameliorates virtually all of the cardiac fibrosis.¹⁵ We have also observed that cardiotonic steroids directly stimulate cardiac fibroblasts to produce increased amounts of collagen through the Na/K-ATPase signal cas-

Received July 28, 2009; first decision August 23, 2009; revision accepted October 6, 2009.

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DOI: 10.1161/HYPERTENSIONAHA.109.140038

Table 1. Effects of Spironolactone on Physiological Measurements After PNx or Infusion of MBG

Group	Sham (n=8)	S (n=8)	PNx (n=10)	PNx+S (n=6)	MBG (n=8)	MBG+S (n=6)
Systolic BP						
SBP 0 wk, mm Hg	119±3	108±4*	112±5	115±3	118±3	114±2
SBP 1 wk, mm Hg	123±4	101±4*	160±4†	144±5*‡	151±5†	138±1*¶
SBP 2 wk, mm Hg	125±3	112±3*	185±6†	157±4†§	165±8†	129±4¶
SBP 3 wk, mm Hg	122±3	101±3*	183±3†	148±3†§	154±6†	125±7¶
SBP 4 wk, mm Hg	125±1	99±3	169±2†	130±3§	162±1†	123±1¶
Heart weight						
BW, g	469±12	460±9	460±18	462±11	503±16	502±14
HW, g	1.32±0.03	1.30±0.2	1.54±0.06†	1.30±0.03§	1.68±0.06†	1.36±0.05¶
HW/BW, ×10 ³	2.95±0.05	2.82±0.09	3.46±0.08*	2.83±0.07§	3.40±0.09*	2.70±0.09¶
Plasma measurements						
Creatinine, mg/dL	0.39±0.02	0.38±0.03	0.60±0.07*	0.63±0.05*	0.41±0.03	0.38±0.02
Potassium, mEq/L	5.8±0.3	5.6±0.4	6.3±0.3	6.6±0.4	6.2±0.3	6.1±0.3
MBG, pmol/L	371±43	321±86	903±135†	994±154†	887±140†	762±98†
Aldosterone, pg/mL	280±33	249±55	1890±277†	1750±292†	245±20	261±45

S indicates spironolactone; SBP, systolic BP measured in conscious animals with a tail cuff on weekly intervals after surgery; BW, body weight; HW, heart weight determined at the time of euthanasia.

* $P < 0.05$ vs sham.

† $P < 0.01$ vs sham.

‡ $P < 0.05$ vs PNx.

§ $P < 0.01$ vs PNx.

¶ $P < 0.05$ vs MBG.

¶ $P < 0.01$ vs MBG.

cade. On the basis of these observations, we speculated that spironolactone might antagonize the signaling of cardiotoxic steroids and have an ameliorative effect in the partial nephrectomy model of uremic cardiomyopathy. To test this hypothesis, the following studies were performed.

Methods

Animals

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

Experimental Groups

Rats weighing between 250 and 300 g were divided into 6 groups with 6 to 12 rats surviving surgery in each group. The first group was subjected to sham surgery, whereas the second group was subjected to fifth/sixth partial nephrectomy (PNx), as described previously.¹⁵ In the third group, sham surgery was performed, and a minipump (model 2004, Alzet) infusing spironolactone (Sigma-Aldrich) at 20 mg/kg per day was inserted SC through a flank incision.¹⁷ The fourth group was subjected to PNx with a minipump infusing spironolactone at 20 mg/kg per day. The fifth group was subjected to sham surgery with a minipump infusing MBG at 10 µg/kg per day, whereas the sixth group was subjected to sham surgery with minipumps infusing MBG at 10 µg/kg per day and spironolactone at 20 mg/kg per day.

Blood Pressure, Cardiac Physiological, and Other In Vivo Measurements

Conscious blood pressure (BP) was measured by the tail-cuff method. Some anesthetized rats were instrumented with a Millar 2.0F catheter placed into the carotid artery for measurement of left

ventricular pressure and volume before euthanasia. MBG, aldosterone, and creatinine were also measured on blood drawn before euthanasia. All of these methods have been described previously.^{16,18}

Isolation of Cardiac Fibroblasts

Isolation of cardiac fibroblasts was carried out as described previously by Brilla et al¹⁹ with modifications as reported previously.¹⁶

Western Blot Analysis

Western Blot analysis was performed on proteins isolated from cell lysates or from tissue homogenates as reported previously.^{14–16,20}

Histology

Trichrome staining was performed on left ventricular tissues and fibrosis quantified as we have reported previously.¹⁵

Collagen Synthesis

(³H)Proline incorporation in cardiac fibroblasts was performed as described previously.^{16,19}

(³H)Ouabain Binding

Ouabain binding studies were performed as described previously.²¹

Statistical Analysis and Expanded Methods

Statistical analysis and details regarding methods are provided in an online Data Supplement (available at <http://hyper.ahajournals.org>).

Results

Spironolactone Attenuates the Development of Cardiomyopathy After Fifth/Sixth Nephrectomy

Spironolactone at doses of 20 mg/kg per day reduced systolic BP modestly but did not significantly alter cardiac physiological function or heart weight compared with control animals subjected to sham surgery (Table 1). Both MBG

Table 2. Effects of Spironolactone on Left Ventricular Functional Measurements After PNx or Infusion of MBG

Group	Sham (n=12)	S (n=10)	PNx (n=12)	PNx+S (n=8)	MBG (n=10)	MBG+S (n=8)
EDV, μL	193 \pm 15	197 \pm 22	129 \pm 9†	200 \pm 15§	147 \pm 11*	207 \pm 18¶
ESV, μL	95 \pm 9	97 \pm 5	39 \pm 7†	85 \pm 10§	57 \pm 6†	99 \pm 12
SV, μL	115 \pm 8	120 \pm 16	106 \pm 10	109 \pm 9	98 \pm 9	108 \pm 11
EF, %	57 \pm 2	55 \pm 3	76 \pm 3†	54 \pm 8†§	64 \pm 3*	60 \pm 4
Tau, ms	11.9 \pm 0.7	10.6 \pm 0.9	17.6 \pm 2.0*	13.3 \pm 1.2‡	12.8 \pm 0.9	9.9 \pm 1.4
EDPVR, mm Hg/ μL	0.031 \pm 0.004	0.031 \pm 0.001	0.046 \pm 0.006*	0.027 \pm 0.005‡	0.042 \pm 0.004*	0.031 \pm 0.005

S indicates spironolactone; EDV, end-diastolic volume; ESV, end-systolic volume; SV, stroke volume; EF, ejection fraction; Tau, time constant for isovolumic relaxation; EDPVR, slope of the regression line fit to the end-diastolic pressure and volume data generated by inferior vena caval constriction.

* P <0.05 vs sham.

† P <0.01 vs sham.

‡ P <0.05 vs PNx.

§ P <0.01 vs PNx.

|| P <0.05 vs MBG.

¶ P <0.01 vs MBG.

infusion (10 $\mu\text{g}/\text{kg}$ per day) and PNx surgery resulted in substantial increases in BP, as well as the heart weight. Coadministration of spironolactone with either MBG or PNx lowered conscious BP, as well as completely attenuated the increases in heart weight. PNx resulted in similar plasma concentrations of MBG as MBG infusion alone, whereas substantial increases in plasma aldosterone concentrations were restricted to those animals subjected to PNx. The addition of spironolactone did not significantly affect either aldosterone or MBG concentrations. These data are summarized in Table 1.

In addition, we examined cardiac function using a pressure- and volume-sensing catheter placed into the left ventricle under anesthesia, as reported previously.^{16,18} We observed that PNx and MBG infusion both induced diastolic dysfunction, as assessed by increase in the time constant for isovolumic ventricular relaxation (τ , significantly increased only in the PNx group), as well as the slope of the pressure-volume relationship at end diastole assessed during inferior vena cava constriction. Additional evidence for this diastolic dysfunction could be inferred from the decreases in end diastolic volume seen in the PNx and MBG groups. These changes were significantly attenuated by concomitant spironolactone therapy in both PNx- and MBG-treated animals (Table 2).

Spironolactone Attenuates Cardiac Fibrosis

We assessed this fibrosis with both histological analysis (trichrome staining, Figure 1A and 1B), as well as collagen 1 content determined by Western blot (Figure 1C). Both PNx and MBG infusion caused dramatic increases in cardiac scarring and collagen content. Coadministration of spironolactone essentially prevented the cardiac fibrosis (Figure 1A through 1C).

Spironolactone and Canrenone Attenuate Procollagen Expression and Radiolabeled Proline Incorporation Induced by MBG in Primary Culture of Cardiac Fibroblasts

Using cultured cardiac fibroblasts, MBG increased procollagen 1 expression determined with Western blot (Figure 2) and

collagen production determined by radiolabeled proline incorporation into the cellular matrix (Table 3). MBG also increased proline incorporation into the supernatant to a similar degree as before (data not shown). Aldosterone applied at concentrations ranging from 1 to 100 nM did not significantly affect radiolabeled proline incorporation (Table 3) or procollagen expression determined by Western blot (data not shown). Both spironolactone (100 nM and 1 $\mu\text{mol}/\text{L}$) and canrenone (100 nM and 1 $\mu\text{mol}/\text{L}$) attenuated proline incorporation at baseline and blocked MBG stimulation of collagen production (Table 3). This was also confirmed with Western blot (Figure 2A and 2B). Interestingly, increasing the amount of MBG to 100 nM could overwhelm the inhibition of spironolactone (100 nM) or canrenone (100 nM), as demonstrated in Figure 2A and 2B.

To examine the effect of canrenone on actual MBG signaling through the Na/K-ATPase, we first examined activation of ERK1/2 (also called p42-44 mitogen activated protein kinase). We found that MBG treatment stimulated increases in phosphorylated ERK1/2; the increases seen with 1 nM MBG could be prevented by coincubation with canrenone at 100 nM, but 100 nM MBG overwhelmed most of the inhibition by this dose (100 nM) of canrenone (Figure 2C). We further examined MBG signaling by examining the effects of MBG with and without canrenone on the translocation of protein kinase C (PKC)- δ , because we have demonstrated previously that MBG induces the translocation of PKC- δ to the nucleus and that this translocation appears necessary for increases in collagen synthesis.²² MBG (1 nM) led to a substantial decrease in cytosolic but an increase in nuclear PKC- δ .²² Canrenone (100 nM) alone appeared to actually increase the amount of PKC- δ in the cytosol and decrease PKC- δ in the nucleus, whereas the coincubation of cells with MBG (1 nM) and canrenone (100 nM) did not substantially change the distribution of PKC- δ compared with controls (Figure 2D).

Spironolactone and Canrenone Inhibit Ouabain Binding

To further examine the interactions among spironolactone, canrenone, and cardiotonic steroids, we performed experi-

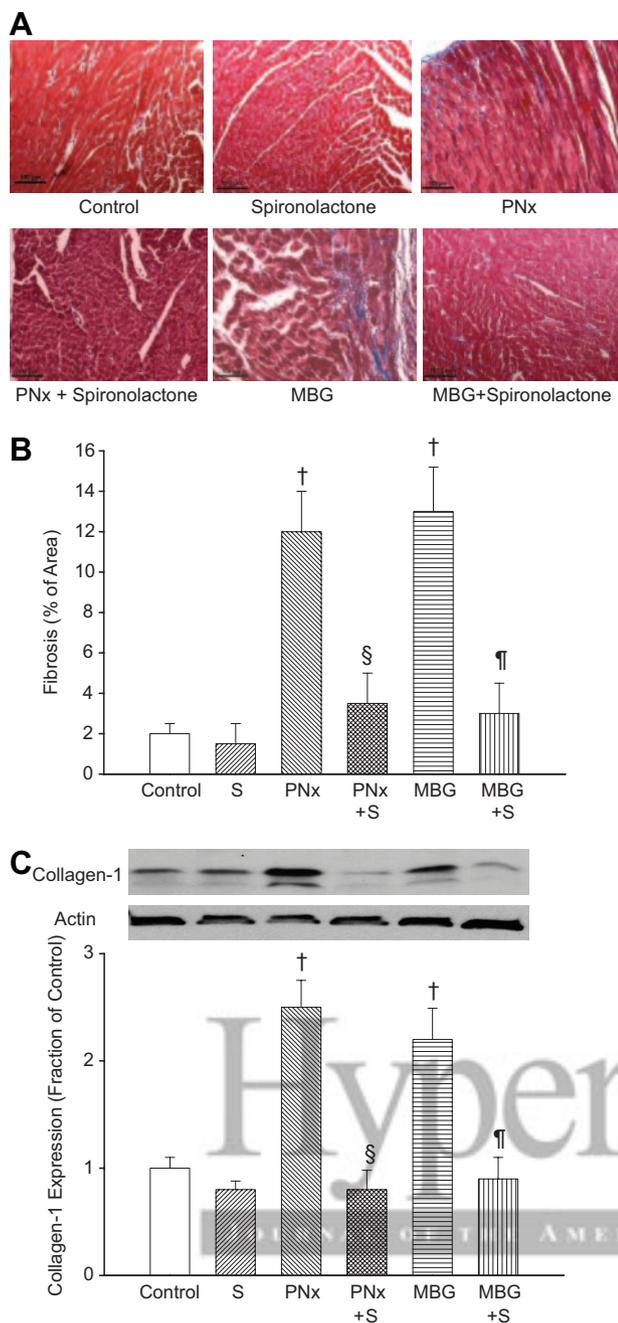


Figure 1. A, Representative trichrome-stained photomicrographs obtained from cardiac tissue derived from the different experimental groups. B, Amount of fibrosis expressed as the mean \pm SEM measured using computer-assisted morphometry, as we have described previously.¹⁵ C, Representative (top) and quantitative analysis of collagen 1 (mean \pm SEM) Western blots performed on cardiac tissues from the different groups. Actin was used to control loading. Control refers to animals subjected to sham surgery (n=8); S refers to spirinolactone infusion using minipumps (n=8); PNx refers to partial nephrectomy (n=10); and MBG refers to MBG infusions using minipumps (n=8). PNx S (n=6) and MBG+S (n=6) refer to combining those 2 maneuvers. † P <0.01 vs control, § P <0.01 vs PNx, ¶ P <0.01 vs MBG.

ments in LLC-PK1 cells, as well as with the Na/K-ATPase isolated from a porcine kidney. The reason that we moved to porcine samples was because the $\alpha 1$ isoform from the pig binds ouabain quite tightly, allowing for easy measurement of

ouabain binding. Because no radiolabeled form of MBG was available to us, we purchased (3 H)ouabain and performed binding studies as described in the Methods section. We found that both canrenone and spirinolactone (at high concentrations, eg, 50 and 100 μ mol/L) significantly shifted binding of ouabain to LLC-PK1 cells (representative data in Figure 3A; quantitative curve fitting data shown in Table S1, please see the online Data Supplement at <http://hyper.ahajournals.org>). On the basis of analysis of these data, both canrenone and spirinolactone increased the apparent dissociation constants for ouabain without significantly affecting the B_{max} (Table S1). This was confirmed for canrenone using the purified Na/K-ATPase isolated from porcine kidney or heart tissues (Table S1). A Scatchard plot of these data also demonstrates that the slope of the lines fit to data were shifted dramatically by the addition of canrenone, whereas the intercept with the x axis was not significantly affected (Figure 3B), further illustrating the competitive nature of the interaction between canrenone and cardiotonic steroids.

Discussion

Cardiac fibrosis complicates a number of clinical cardiomyopathies, including those seen with renal failure.^{23–26} Spirinolactone improves outcomes in patients with dilated cardiomyopathy^{27,28} and attenuates fibrosis in several experimental cardiomyopathies.^{17,29–32} It also appears that spirinolactone or canrenone may ameliorate renal injury^{33–35} and potentially attenuate cardiac hypertrophy in experimental renal failure.²⁹

The beneficial effects of spirinolactone have been attributed to antagonism of the profibrotic effects of aldosterone in early studies, because spirinolactone and its major metabolite, canrenone, are known to compete with aldosterone at the level of the mineralocorticoid receptor.³⁶ That said, it should be stressed that much of the data supporting a profibrotic effect of aldosterone are represented by the beneficial effects of spirinolactone or other mineralocorticoid antagonists.^{29,31,32,37} In vivo, the profibrotic effects of aldosterone appear to be confined to conditions where extracellular volume is expanded.^{30,38} When studied in vitro using cardiac fibroblasts, a stimulation of collagen production has been noted by some authors,¹⁹ but many workers report having observed no stimulation,^{39–41} similar to that which we observed in this report. This complex area has been the topic of a number of recent reviews.^{42–44}

In the current study, we observed that spirinolactone therapy markedly attenuated the cardiomyopathy induced by either experimental renal failure induced by partial nephrectomy or by administration of MBG in a manner designed to mimic the increases in the circulating levels of this hormone observed after partial nephrectomy.^{15,16} Plasma MBG concentrations have been reported in normal subjects typically within the 300 to 400 pM range, depending on diet.⁴⁵ The concentrations seen in vivo in experimental animals that we observed in this study (900 pM) were slightly higher than that which we have reported previously,^{15,16} but the ratio of experimental to sham values was similar. The cardiomyopathy is characterized by diastolic dysfunction and cardiac hypertrophy. The diastolic dysfunction, in particular, is evidenced by the decreases in end-diastolic and end-systolic

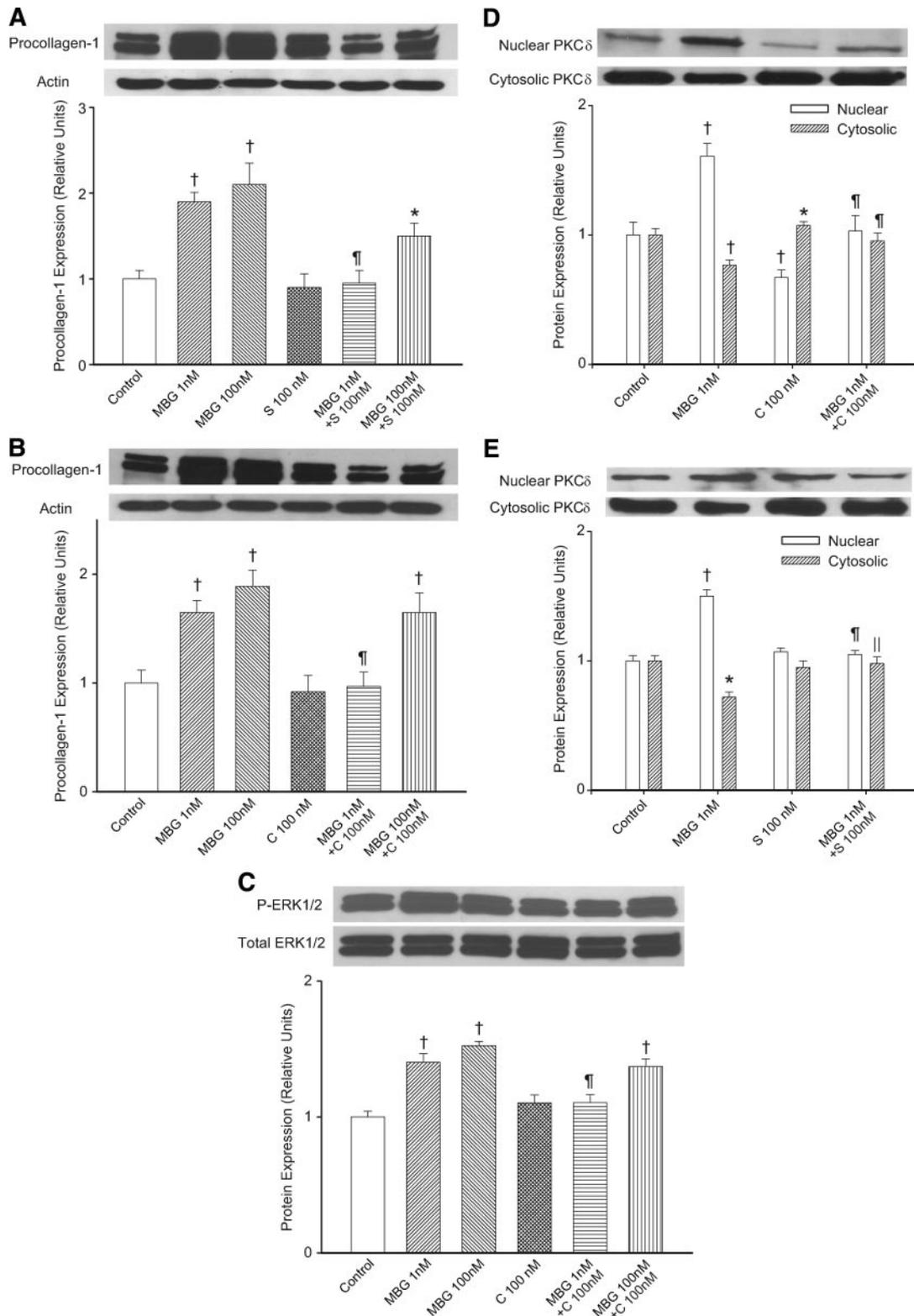


Figure 2. A, Representative Western blot against procollagen 1 derived from cardiac fibroblasts treated with MBG (1 or 100 nM), spironolactone (100 nM), or a combination with the corresponding quantitative data shown as the mean \pm SEM of 6 experiments shown below. B, Representative Western blot against procollagen 1 derived from cardiac fibroblasts treated with MBG (1 or 100 nM), canrenone (100 nM), or a combination with the corresponding quantitative data shown as the mean \pm SEM of 6 experiments shown below. C, Representative phosphorylated ERK1/2 (P-ERK1/2) and total ERK 1/2 (Total ERK1/2) blots as well as the quantitative ratio of P-ERK1/2/Total ERK1/2 (mean \pm SEM; n=9 each group) in cells treated similarly to B but harvested at 2 hours. D, The effects of MBG (1 nM), canrenone (100 nM), or a combination of the 2 treatments (15 minutes exposure) on PKC- δ expression in the cytosol and nucleus. E, The effects of MBG (1 nM), spironolactone (100 nM), or a combination of the 2 treatments (15-minute exposure) on PKC- δ expression in the cytosol and nucleus. For both D and F, representative Western blots are shown in the top portion of the panel with quantitative data (mean \pm SEM of 5 determinations) shown below. * P <0.05 and $\dagger P$ <0.01 vs control; $\parallel P$ <0.05; ∇P <0.01 vs MBG.

Table 3. Effect of MBG, Spironolactone, and Canrenone on Radiolabeled Proline Incorporation

Group (N)	Proline Incorporation (Fraction of Control Values)
Control (64)	1.00±0.02
MBG, 1 nmol/L (20)	1.58±0.06*
MBG, 10 nmol/L (36)	1.67±0.04*
MBG, 100 nmol/L (8)	1.64±0.10*
Aldosterone, 1 nmol/L (24)	1.04±0.03
Aldosterone, 10 nmol/L (10)	0.93±0.08
Aldosterone, 100 nmol/L (24)	0.97±0.04
Spironolactone, 100 nmol/L (20)	0.85±0.03*
Spironolactone, 1 μmol/L (32)	0.80±0.05*
Canrenone, 100 nmol/L (12)	0.82±0.04*
Canrenone, 1 μmol/L (12)	0.66±0.05*
MBG, 1 nmol/L+spironolactone, 100 nmol/L (20)	0.98±0.04†
MBG, 1 nmol/L+spironolactone, 1 μmol/L (20)	0.80±0.03*†
MBG, 1 nmol/L+canrenone, 100 nmol/L (12)	0.76±0.06*†
MBG, 1 nmol/L+canrenone, 1 μmol/L (12)	0.65±0.07*†
MBG, 100 nmol/L+spironolactone, 100 nmol/L (8)	1.65±0.05*
MBG, 100 nmol/L+canrenone, 100 nmol/L (8)	1.72±0.06*

* $P < 0.01$ vs control.† $P < 0.01$ vs MBG, 1 nmol/L.

volumes, as well as increases in τ values and the pressure-volume relationship at end diastole assessed during inferior vena cava constriction, representing impairment of both active and passive relaxation. We note that the magnitude of the diastolic dysfunction did appear to be somewhat more severe in the PNx animals as compared with the MBG infusion group, suggesting that other neurohumoral changes resulting from impaired renal function (eg, increases in parathyroid hormone and abnormalities in vitamin D metabolism) may contribute to the cardiomyopathy induced by PNx.^{26,46} Spironolactone therapy dramatically attenuated these hemodynamic abnormalities, as well as the cardiac growth seen in both the PNx- and MBG-infused groups. We had reported previously that, whereas the creation of partial nephrectomy resulted in marked increases in aldosterone, administration of MBG did not cause significant increases in the circulating concentrations of this hormone.^{15,16} These findings were confirmed in the current study, and it did not appear that administration of spironolactone substantially altered either the plasma concentrations of MBG or aldosterone. Both PNx and MBG resulted in substantial increases in cardiac collagen content and fibrosis, as we have reported previously^{15,16}; spironolactone administration also dramatically attenuated these changes, which have been linked to the passive component of the diastolic dysfunction. Although the administration of spironolactone also attenuated the hypertension seen with both PNx and MBG, other work that we have presented suggests that at least some of the cardiac fibrosis caused by PNx appears to be independent of BP increases.^{14,15,18} Recently, Michea et al⁴⁷ studied the effects of spironolactone on the cardiac alterations induced by experi-

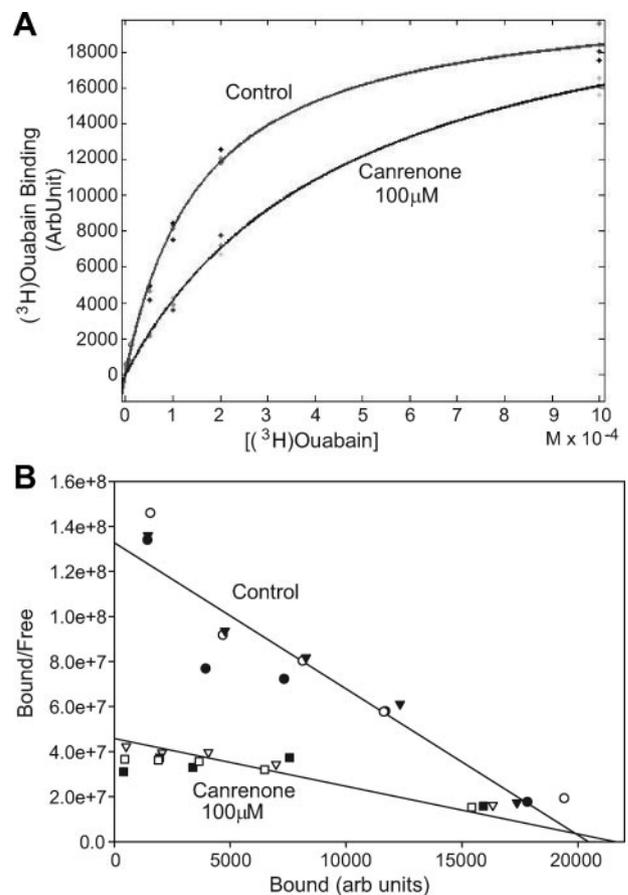


Figure 3. A, (^3H) ouabain binding data expressed as a function of (^3H) ouabain concentration either without (control) or with canrenone at $100 \mu\text{mol/L}$. Curves shown are fit to the average of 3 separate sets of experiments in the control and canrenone groups. The R^2 value for both curve fits exceeded 0.97. B, The same experimental data transformed into bound/free vs bound (Scatchard plot) with regression lines fit to both the control and canrenone data. Note that the slopes of the lines (reflecting the reciprocal of the dissociation constant values) are quite different, whereas the intersections of these lines with the x axis (reflecting the reciprocal of the B_{max} values) occur at essentially the same point. Quantifications of the dissociation constant and B_{max} data were performed using nonlinear regression methods (MATLAB) on data represented in A; these data are presented in the online Data Supplement (Table S1).

mental renal failure. These workers also noted that spironolactone attenuated cardiac growth, as well as oxidant stress, induced by PNx.

Moving to the in vitro setting, we observed that MBG at concentrations similar to those seen in our experimental models of cardiac fibrosis induced increases in collagen production by rat cardiac fibroblasts as assessed by proline incorporation and procollagen 1 expression determined by Western blot, as we have reported previously¹⁶; both spironolactone and its major metabolite, canrenone, prevented these changes. Canrenone also appeared to prevent the signaling of MBG through the Na/K-ATPase, as assessed by ERK1/2 activation and PKC- δ translocation. When we increased MBG to much higher concentrations, the effects of both spironolactone and canrenone on collagen synthesis and ERK1/2 activation could be overwhelmed. Finally, we performed binding studies using (^3H) ouabain in LLC-PK1 cells,

as well as the Na/K-ATPase isolated from porcine kidneys. (³H)ouabain was used rather than MBG because of its easy availability, and cells or enzymes obtained from pig were used because of their high affinity for ouabain. We observed that ouabain binding was inhibited by both spironolactone and canrenone in what appeared to be a competitive manner. In aggregate, our data suggest that spironolactone and its major metabolite, canrenone, appear to competitively inhibit MBG signaling and stimulation of collagen production in vitro, a phenomenon that may explain the beneficial effects of spironolactone in vivo.

The idea that canrenone (or spironolactone) may function as a competitive inhibitor for cardiotonic steroids is not a novel concept. Selye et al⁴⁸ found that spironolactone therapy could dramatically attenuate experimental digitoxin toxicity in the rat. Erdmann et al⁴⁹ demonstrated that canrenone could ameliorate digitalis-induced cardiac arrhythmias in some patients, as well as displace labeled strophanthin from red cell-binding sites. Finotti and Palatini¹¹ suggested that that canrenone interacted with isolated Na/K-ATPase at the same site. Garay et al⁵⁰ demonstrated that canrenone attenuated digitalis-induced inhibition of the NaK-ATPase in human red blood cells, whereas Balzan et al¹⁰ made similar observations in human red blood cells and placenta. Sorrentino et al⁵¹ observed that canrenone could antagonize the vasoconstrictor effects of ouabain. In addition to the aforementioned data suggesting that spironolactone and canrenone can act as classical competitive inhibitors, however, there are also data suggesting that both spironolactone and canrenone may influence signaling through Na/K-ATPase through alteration of its lipid environment.^{52–54}

Perspectives

Our study demonstrates that administration of spironolactone attenuates cardiac fibrosis in both partial nephrectomy and MBG infusion models. Both spironolactone and canrenone appear to competitively antagonize cardiotonic steroid-induced fibroblast stimulation. On the basis of these findings in concert with existing literature, we would propose that MBG may be responsible for some of the cardiac injury that has been attributed to aldosterone and that spironolactone, canrenone, and other so-called mineralocorticoid antagonists may actually produce some of their beneficial effects by antagonism of cardiotonic steroid hormone signaling through the plasmalemmal Na/K-ATPase. If these data are confirmed in humans, this may open up new therapeutic indications for spironolactone, as well as allow for the design of other agents that specifically target cardiotonic steroid-induced processes.

Acknowledgments

We thank Carol Woods for her excellent secretarial assistance.

Sources of Funding

Portions of this work were supported by grants from the National Institutes of Health (HL-67963, HL-36573, HL-071556, and GM-78565), as well as intramural support from the National Institute on Aging Laboratory of Cardiovascular Science.

Disclosures

None.

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Hypertension. published online November 2, 2009;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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Spironolactone Attenuates Experimental Uremic Cardiomyopathy by Antagonizing Marinobufagenin

by

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Short Title: Spironolactone attenuates signaling via Na pump.

Key Words: Cardiomyopathy; Renal Failure, Cardiotonic Steroids; Collagen; Fibrosis

Expanded Methods:

Animals: Male Sprague-Dawley rats were used for all of the studies. All of the 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 Committee (IACUC).

Experimental Groups: Rats weighing between 250 and 300 grams were divided into 6 groups. Pentobarbital (50 mg/kg IP) was used for surgeries or cardiac physiological measurements (*vida infra*). The first group was subjected to sham surgery (SS) while the second group was subjected to 5/6th nephrectomy as previously described¹. In the third group, SS was performed and a minipump (model 2004, Alzet, Palo Alto, CA) infusing spironolactone (Sigma-Aldrich, St. Louis, MO) at 20 mg/kg per day was inserted subcutaneously through a flank incision². The fourth group was subjected to a partial nephrectomy (PNx) with a minipump infusing spironolactone at 20 mg/kg per day. The fifth group was subjected to SS with a minipump infusing MBG at 10 µg/kg per day whereas the sixth group was subjected to SS with minipumps infusing MBG at 10 µg/kg per day and spironolactone at 20 mg/kg per day.

Both before and after surgeries, animals were fed a standard rat chow (Rodent Laboratory Chow 5001, PMI Nutrition International Inc., Brentwood, MO) which contained sodium (amount), potassium (amount). The rat chow also was (amount) protein, (amount) carbohydrate and fat.

Blood Pressure: After surgery, the rats were allowed to recover for 4 weeks with easy access to chow mix food (Rodent Laboratory Chow 5001, PMI Nutrition International Inc., Brentwood, MO) and water. Conscious blood pressure (BP) was measured by the tail cuff method with equipment made by IITC, Inc. (Amplifier model 229, Monitor model 31, Test chamber Model 306; IITC Life Science, Woodland Hills, CA) as previously described^{1,3}. At the end of 4 weeks following surgery, animals were euthanized, their hearts were weighed, and cardiac tissue was prepared for histology and biochemistry as previously described^{1,3,4}.

Cardiac Physiological Measurements: Some anesthetized rats were instrumented with a Millar 2.0-Fr catheter placed into the carotid artery. Left ventricular pressure and volume were recorded under steady state conditions as well as during inferior vena cava constriction. These data were analyzed using the PVAN 3.5 software as previously reported³.

MBG Purity: MBG was isolated from *Bufo Marinus* venom as described previously⁵. The isolated MBG was >99% pure based on high-performance liquid chromatography and mass spectroscopy analysis.

MBG Measurement: Plasma MBG concentration was determined using a competitive enzyme-linked immunosorbent assay (ELISA) as described before^{1,6}. Briefly, the plasma samples (0.5 ml) were extracted on Sep-Pak C-18 columns. The combination of 20% and 80% acetonitril elutes was lyophilized and re-suspended in TBS buffer (Tris 50 mM, NaCl 150 mM, NaN₃ 7.7 mM, pH 7.4). 100 µl of MBG standards or samples were mixed with 100 µl anti-MBG monoclonal antibody. The mixture was then added to MBG-thyroglobulin-coated and 1% BSA-blocked ELISA plate. After 1 h incubation, plates were washed and secondary anti-mouse antibody was added. A fluorescent signal

amplifier, FDP Alkaline Phosphatase, from ANASpec (San Jose, CA) was used to detect the signals after washing out the secondary antibody.

Aldosterone Measurement: Plasma samples were first diluted 10 times and aldosterone concentrations were measured using an EIA kit from Cayman Chemical (Ann Arbor, MI Cat# 10004377). Samples or standards were mixed with equal volume of anti-aldosterone antibody and aldosterone-AChE tracer in a plate coated with anti-mouse antibody. After overnight incubation at 4°C, plates were washed and 200 μ l Ellman's reagent was added to develop the signals. OD value was measured after 90 minutes at 410 nm¹.

Creatinine Measurement: Plasma creatinine was measured with colorimetric method using a commercial kit from Teco Diagnostics (Anaheim, CA, Cat# C515-490). Creatinine standards or plasma samples were mixed with the picric acid reagent and creatinine buffer reagent provided with the kit. The OD value at 510 nm was measured immediately after and at 15 min. The differences between the two time points were used to calculate the creatinine concentrations.

Isolation of Cardiac Fibroblasts: Isolation of cardiac fibroblasts was carried out as previously described by Brilla and coworkers⁷ with modifications as previously reported³. Briefly, male Sprague Dawley rats weighing 250-300 grams were used to obtain fibroblast from the hearts. The rats were anesthetized with pentobarbital (50 mg/kg), and their hearts were removed and perfused under sterile condition via the ascending aorta with Joklik's medium (Sigma-Aldrich, St. Louis, MO) in a modified Langendorff apparatus. After 5 min of perfusion, the perfusate was placed in Joklik's medium containing 0.1% collagenase type 2 (Worthington Biochemical, Lakewood, NJ) and 0.1% BSA which was circulated for 15-25 min until the heart became flaccid. Ventricles were excised and finely cut, and shaken in Joklik's modified medium with 0.1% collagenase and 0.1% BSA for 15 min. Cells /tissue suspension was allowed to settle for 15 min and was centrifugated at 500 rpm for 10 min. The supernatant then was centrifugated at 1500 rpm for 15 min. The resulting pellet was suspended in DMEM supplemented with antibiotics (penicillin/streptomycin/fungizone) plus 15% FBS (Hyclone, Logan, UT) and seeded onto plates and incubated for 1hr. Unattached cells were removed, and the attached fibroblasts cells were allowed to grow until confluence and then trypsinized and passaged once at 1:3 dilution. Cells were allowed to grow confluent prior to use for experimental purposes. All cells used in these experiments were from passage one unless otherwise specified.

Western Blot Analysis: Western Blot analysis was performed on proteins isolated from cell lysates or from tissue homogenates as previously reported^{1, 3, 4, 8}. 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 when looking for procollagen expression and for 15 minutes when looking for phosphorylated proteins expression such as ERK1/2. The cells were washed with phosphate buffered saline (BPS) twice and exposed to lysis buffer. As 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 centrifugated at 12,000 G for 10 min at 4°C. 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, obtained from cell lysates or tissue

homogenates, were resolved on an SDS-PAGE using Precast Ready Gels 4-15 % Tris-HCl, purchased from Bio-Rad (Hercules, CA). 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-type1 collagen antibody (Southern Biotech, Birmingham, AL) was used to probe for procollagen-1 (Invitrogen/ Biosource, Carlsbad, CA. Anti-ERK1/2 polyclonal antibody, anti-phospho-ERK1/2 monoclonal antibody, and secondary anti-goat and anti rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). For detection, we used ECL and ECL plus purchased from Amersham Biosciences (Piscataway, NJ). Loading conditions were controlled for using either tubulin or actin (mouse monoclonal, Santa Cruz).

Determination of protein kinase C isoform delta (PKC δ) in the nucleus and cytosol: Cardiac fibroblasts were grown to confluence and starved for 18-24hrs. Cells were treated with MBG (1 nM) and/or canrenone (100 nM) added to the media. After 15min, cells were washed with ice-cold PBS and both cytosolic and nuclear extracts were prepared from treated and untreated cells. PKC δ in the cytosol and nucleus was determined by performing Western blot as described previously⁹.

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

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 (1 μ Ci/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 μ L 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^{3,7}.

(³H)Ouabain binding: Ouabain binding studies were performed in a porcine cell line (LLC-PK1 cells) as previously described¹⁰. Briefly, cells were seeded into 12-well plates, cultured to 90% confluence and then serum starved overnight. Afterward, cells were rinsed, incubated in K⁺ free Krebs solution in the presence of 20 μ M monensin to clamp intracellular Na⁺ and prevent recycling of the Na/K-ATPase. At the end of 15 minutes of incubation, cells were washed 4 times with ice-cold K⁺ free Krebs solution, solubilized in 0.1N NaOH/0.2% SDS and measured with a scintillation counter.

Statistical analysis: Data presented are mean \pm 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 correction¹¹. Statistical analysis was performed using Matlab™ software.

Expanded Results:

Spirolactone and canrenone inhibit ouabain binding: To further examine the interactions between spironolactone, canrenone and cardiotonic steroids, we performed experiments in LLC-PK1 cells as well as with the Na/K-ATPase isolated from porcine kidney. The reason we moved to porcine samples was because the alpha1 isoform from the pig binds ouabain quite tightly allowing for easy measurement of ouabain binding. As no radio-labeled form of MBG was available to us, we purchased (³H)ouabain and performed binding studies as described in the methods. We found that both canrenone and spironolactone (at high concentrations) significantly shifted binding of ouabain to LLC-PK1 cells (representative data in figure 3a, quantitative curve fitting data shown in table S1). Based on analysis of these data, both canrenone and spironolactone increased the apparent Kd for ouabain without significantly affecting the Bmax (table S1). This was confirmed for canrenone using the purified Na/K-ATPase isolated from porcine kidney and porcine heart (Table S1). A Scatchard plot of these data also demonstrates that the slope of the lines fit to data were shifted dramatically by addition of canrenone whereas the intercept with the X axis was not significantly affected (Figure 3b), further illustrating the competitive nature of the interaction between canrenone and cardiotonic steroids.

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Table S1: Effect of Spironolactone and Canrenone on (³H)Ouabain Binding

Group (N)	Bmax (Fraction Control)	Kd (nM)
LLCPK1 Cells		
Control (24)	1.00 +/- 0.07	165 +/- 12
Canrenone 10 uM (3)	1.18 +/- 0.12	220 +/- 13†
Canrenone 50 uM (3)	1.02 +/- 0.08	297 +/- 34†
Canrenone 100 uM (6)	1.11 +/- 0.05	452 +/- 52 †
Spironolactone 1 uM (3)	1.07 +/- 0.09	169 +/- 14
Spironolactone 10 uM (3)	1.04 +/- 0.12	175 +/- 35
Spironolactone 100 uM (6)	1.07 +/- 0.11	233 +/- 65†
Isolated Porcine Na/K-ATPase from Kidney Tissue		
Control (21)	1.00 +/- 0.02	66 +/- 3
Canrenone 10 uM (3)	1.03 +/- 0.04	58 +/- 4
Canrenone 50 uM (3)	0.98 +/- 0.05	78 +/- 5*
Canrenone 100 uM (6)	0.97 +/- 0.07	151 +/- 4†
Spironolactone 10 uM (3)	0.95 +/- 0.06	61 +/- 4
Spironolactone 100 uM (3)	0.96 +/- 0.06	95 +/- 5†
Isolated Porcine Na/K-ATPase from Heart Tissue		
Control (15)	1.00 +/- 0.03	55 +/- 4
Canrenone 10 uM (3)	1.05 +/- 0.04	52 +/- 5
Canrenone 100 uM (6)	1.04 +/- 0.06	148 +/- 14†
Spironolactone 10 uM (3)	0.98 +/- 0.02	68 +/- 5
Spironolactone 100 uM (3)	1.06 +/- 0.04	95 +/- 4†

Controls were performed with each dose of canrenone or spironolactone. Because the LLC PK1 cell density varied from experiment to experiment or the precise amount of enzyme was also variable, Bmax data is expressed as a fraction of control. Kd and Bmax measurements were performed fitting experimental data to the function $Y = \frac{B_{max} * (X)}{Kd + X}$ where Y is (³H) ouabain binding and X is the concentration of ouabain. * p < 0.05 vs Control, † p < 0.01 vs Control.