

# Central Role for the Cardiotonic Steroid Marinobufagenin in the Pathogenesis of Experimental 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 are also known to have increases in the circulating concentrations of the cardiotonic steroid marinobufagenin (MBG). On this background, we hypothesized that elevations in circulating MBG may be involved in the cardiomyopathy. First, we observed that administration of MBG (10  $\mu\text{g}/\text{kg}$  per day) for 4 weeks caused comparable increases in plasma MBG as partial nephrectomy at 4 weeks. MBG 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 MBG infusion and partial nephrectomy. Next, rats were actively immunized against a MBG-BSA conjugate or BSA control, and partial nephrectomy was subsequently performed. Immunization against MBG attenuated the cardiac hypertrophy, impairment of diastolic function, cardiac fibrosis, and systemic oxidant stress seen with partial nephrectomy without a significant effect on conscious blood pressure. These data suggest that the increased concentrations of MBG are important in the cardiac disease and oxidant stress state seen with renal failure. (*Hypertension*. 2006;47:488-495.)

**Key Words:** cardiomyopathy ■ sarcoplasmic reticulum ■ cardiotonic agents ■ fibrosis ■ renal failure ■ reactive oxygen species

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.<sup>1</sup> 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.<sup>2,3</sup>

The partial nephrectomy model in the rat has been used to simulate experimental uremia to study the cardiac abnormalities that accompany renal failure.<sup>4</sup> A number of factors, including volume overload, have been implicated in the pathogenesis of the cardiac disease in this model (reviewed in Reference 5). We have observed that cardiac myocytes isolated from rats subjected to partial (ie, five-sixth) 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.<sup>6</sup> 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.<sup>7</sup> Recent work has established that the cardiotonic steroid marinobufagenin (MBG) induces natriuresis and, in susceptible rat strains, increases blood pressure (BP).<sup>8,9</sup> 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.<sup>10,11</sup>

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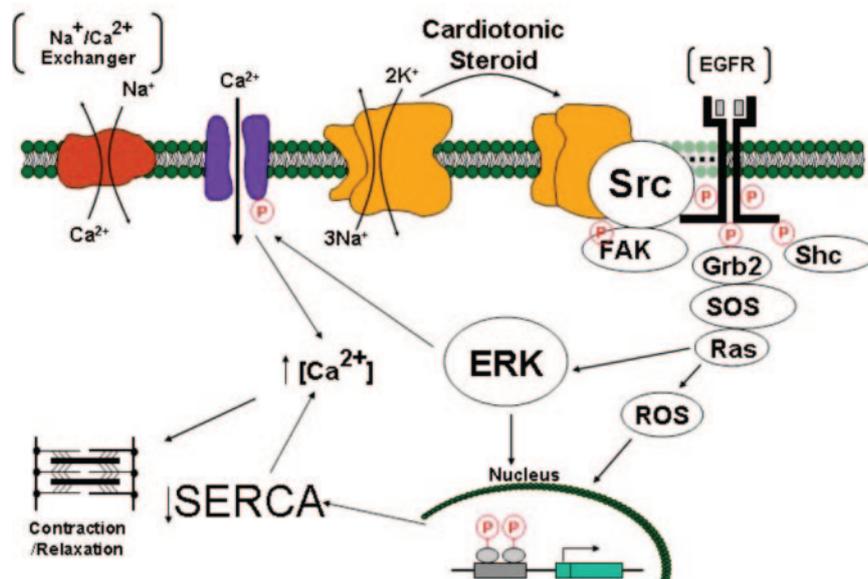
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**Figure 1.** Schematic depicting sodium pump signaling in cardiac myocytes. In the presence of a cardiotoxic steroid, Na/K-ATPase is converted to a signal transducer, which complexes with Src and the epidermal growth factor receptor. A signal cascade 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.<sup>15–19</sup>



Interestingly, investigators postulated a role for endogenous natriuretic substances in the pathobiology of uremia decades before their identification.<sup>12</sup> Our group and others have observed that cardiotoxic steroids induce signaling through the plasmalemmal Na/K-ATPase, which resides in caveolae.<sup>13,14</sup> This signaling requires the generation of reactive oxygen species, has genomic effects that can be attributable to the modulation of transcription factors, including SP-1, and induces hypertrophic changes in both neonatal and adult cardiac myocytes *in vitro*<sup>15–19</sup> (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.<sup>20</sup> 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 before partial nephrectomy. After performing these maneuvers, physiological, morphological, and biochemical assays were performed.

## Methods

### Animals

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

### Experimental Groups

Rats that 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 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 that were immunized against MBG-BSA conjugate before partial nephrectomy are referred to as PNx-IM ( $n=18$ ).

### MBG Infusion

MBG was isolated from toad (*Bufo Marinus*) venom as described previously.<sup>21</sup> The isolated MBG was >99% pure based on high-performance liquid chromatography and mass spectroscopy analysis. MBG was infused for a period of 4 weeks at 10  $\mu\text{g}/\text{kg}$  per day with an osmotic minipump (Alzet Model 2004, Durect Corp). The stability of MBG for 4 weeks at 37°C was confirmed by comparable inhibition of <sup>86</sup>Rb uptake in LLCPK1 cells at  $2.5 \times 10^{-7}$  and  $1 \times 10^{-6}$  M concentrations as MBG prepared immediately, as well as by mass spectroscopy analysis.

### Experimental Renal Failure

Partial nephrectomy (five-sixth nephrectomy) was induced by removal of the right kidney and selective infarction of two-thirds of the left kidney with silk ligatures as described previously.<sup>6</sup>

### Immunization Against MBG

Rats were immunized with an MBG-BSA conjugate and subjected to partial nephrectomy. The immunization schedule was 3 weekly injections (250  $\mu\text{g}/\text{kg}$  per week SQ) in complete Freund's adjuvant before the partial nephrectomy with a last boost at the time of surgery. This regimen, which has been used previously,<sup>22</sup> induced high titers of antibodies ( $>1:10\,000$ ) to MBG. These antibodies had high affinity to MBG ( $4.7 \times 10^9$  to  $5.5 \times 10^8$ ) and very little cross-reactivity ( $\ll 1\%$ ) to aldosterone, ouabain, digoxin, bufalin, and progesterone.

### Hemodynamics

BP was measured once a week by the tail-cuff method<sup>23</sup> in conscious, restrained rats with equipment made by IITC, Inc (Amplifier model 229, Monitor model 31, Test chamber Model 306; IITC Life Science) as described previously.<sup>20</sup> Before sacrifice at 4 weeks, animals had ventricular pressures determined by placement of a 2F Millar Microtip Catheter Transducer (Millar Instruments Inc) 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). The values of left ventricular end-diastolic pressure (LVEDP), 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.<sup>24</sup>

**TABLE 1. Effects of MBG on Various Biochemical and Functional Parameters**

Variable	Sham	PNx	MBG	PNx-IM
Plasma [MBG], pmol/L	359±16	564±36†	546±34†	430±36‡
Urinary MBG excretion (U <sub>MBG</sub> V), pmol/24 h	31.3±2.3	60.2±4.5†	49.1±3.5†	44.7±4.0*‡
Plasma [OLC], pmol/L	428±53	437±43	493±41	528±38
Urinary OLC excretion (U <sub>OLC</sub> V), pmol/24 h	11.7±1.5	11.4±1.9	10.6±0.9	11.1±1.1
Plasma creatinine, mg/dL	0.30±0.03	0.95±0.12†	0.52±0.07	0.95±0.13†
Hematocrit, %	44.5±0.8	38.8±1.3†	44.6±0.9	41.1±0.7†
Plasma parathyroid hormone, pg/mL	41±6	126±17*	41±9	140±26*
Plasma aldosterone, pg/mL	191±55	1780±371†	322±38	2207±474†

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; ‡*P*<0.05 vs PNx; and §*P*<0.01 vs PNx.

### Rat Echocardiography

At the end of 4 weeks, 2D and M-mode echocardiographic studies were performed using a Philips Sonos 5500 cardiovascular ultrasound imaging system (Philips Medical Systems) 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 described previously by Litwin et al.<sup>25</sup>

### Measurement of MBG and Ouabain-Like Compound

MBG and ouabain-like compound (OLC) in plasma and urine was determined at 4 weeks after extraction with C-18 columns as described previously.<sup>11</sup> An expanded Methods section is available online at <http://hyper.ahajournals.org>.

### Western Blot Analysis

At the 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<sup>6</sup> using SDS-PAGE gels (Ready Gel, BioRad).

### 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).<sup>26,27</sup> Total plasma malondialdehyde was measured spectrophotometrically using the Bioxytech MDA-586 kit (Oxis Research).

### 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 van Hardeveld<sup>28</sup> was used with minor modifications as described previously.<sup>6</sup>

### Miscellaneous Blood Analyses

Hematocrit was measured from whole blood collected in heparinized capillary tubes and spun on a microhematocrit centrifuge (Fisher Scientific). Plasma creatinine was determined spectrophotometrically with a colorimetric end point assay (Teco Diagnostics). Plasma aldosterone levels were measured by ELISA (Cayman). Plasma parathyroid hormone was assayed using the Rat Intact PTH ELISA kit (Immutopics). All of the analyses were performed on blood collected upon sacrifice at 4 weeks.

### Histology and Fibrosis Scoring

An expanded section is available online.

### Statistical Analysis

Data presented are mean±SEM. 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<sub>MBG</sub>V) at 4 weeks after surgery compared with control rats (Table 1). Infusion of MBG alone induced comparable increases in plasma [MBG] and U<sub>MBG</sub>V as partial nephrectomy. Immunization against MBG-BSA in partial nephrectomy animals was associated with a decrease in U<sub>MBG</sub>V compared with partial nephrectomy alone. Neither plasma or urine OLC levels were different between sham and partial nephrectomy as we have reported previously<sup>11</sup> nor did we see a significant effect of either MBG supplementation or immunization against MBG on plasma or urine OLC levels (Table 1).

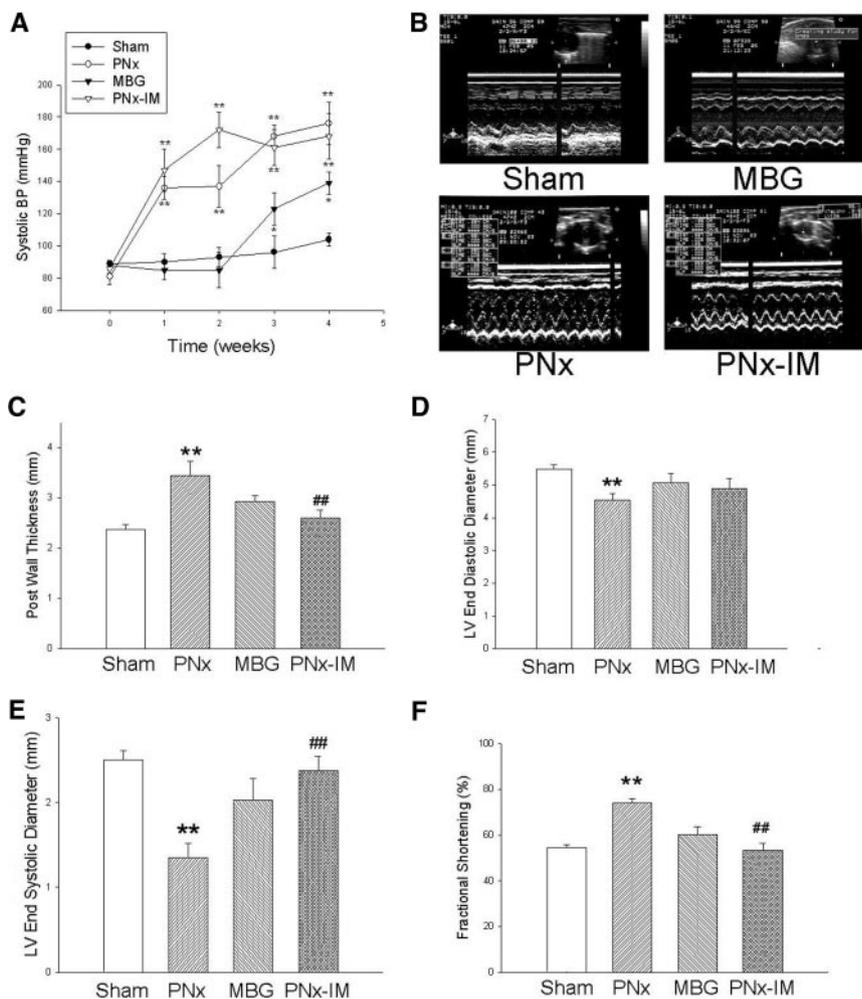
Partial nephrectomy led to marked increases in plasma creatinine and decreases in hematocrit, which were not affected by immunization (Table 1). MBG infusion to sham-operated rats did not significantly alter either of these measurements. Partial nephrectomy induced considerable increases in plasma aldosterone 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, whereas MBG

**TABLE 2. MBG Induces Oxidant Stress In Vivo**

Variable	Sham	PNx	MBG	PNx-IM
Plasma carbonylated protein, pmol/mg protein	171±9	320±20†	378±15†	241±24‡
Left ventricular carbonylated protein, pmol/mg protein	387±23	541±41*	505±22	393±39§
Plasma total malondialdehyde, nM	399±17	571±49†	474±41	428±34

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 2.** MBG produces functional and anatomic changes consistent with cardiac hypertrophy. (a) Systolic BP 4 weeks after sham operation (Sham,  $n=8$ ), partial nephrectomy (PNx,  $n=8$ ), MBG infusion (MBG,  $n=10$ ), or immunization against MBG before partial nephrectomy (PNx-IM,  $n=8$ ). (b) Representative M mode echocardiograms in the 4 groups of rats. (c) Posterior wall thickness, (d) left ventricular end diastolic diameter, (e) left ventricular end systolic diameter, and (f) FS 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.

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 BP during the 4 weeks of observation. MBG infusion alone produced some increases in BP 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 and 2c). These data were consistent with the heart weight data obtained (vide 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 (FS) was also substantially increased (Figure 2f). MBG infusion was not associated with significant changes in wall thickness, chamber size, or FS compared with sham-treated controls. Immunization against MBG ameliorated the echocar-

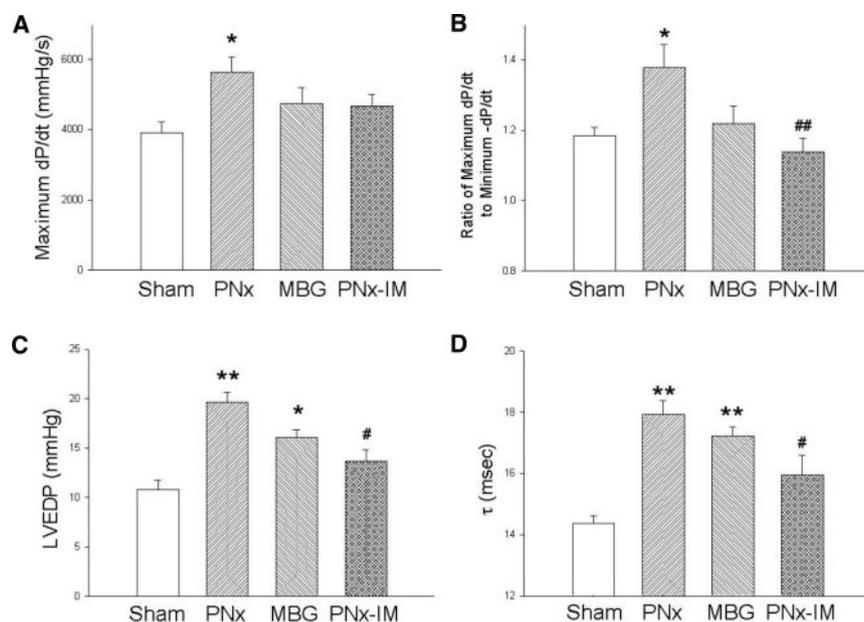
diographic changes noted with partial nephrectomy (Figure 2b through 2f).

After 4 weeks, the animals were anesthetized, and a Millar catheter was introduced into the left ventricle 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 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 (Figure 3a through 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

**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 (ie, most negative rate of pressure change, -dP/dt), (c) left ventricular end diastolic pressure (LVEDP), and (d) time constant of isovolumic relaxation ( $\tau$ ) 4 weeks after sham operation (Sham, n=14), partial nephrectomy (PNx, n=15), MBG infusion (MBG, n=12), or immunization against MBG before 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.



nephrectomy. Partial nephrectomy was associated with activation of extracellular signal regulated kinase (ERK; Figure 4b) and Src (Figure 4c), upregulation of skACT (Figure 4d), as well as downregulation of both the  $\alpha$ 1 and  $\alpha$ 2 isoform of the Na/K-ATPase (Figure 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  $\alpha$ 2 Na/K-ATPase and SERCA2a expression and SERCA function with partial nephrectomy (Figure 4a through 4g).

Partial nephrectomy resulted in marked increases in cardiac fibrosis as assessed by either semiquantitative 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 (Figure 5a through 5c). Partial nephrectomy was associated with marked increases in fibronectin, whereas immunization against MBG markedly attenuated the changes in fibronectin (Figure 5d).

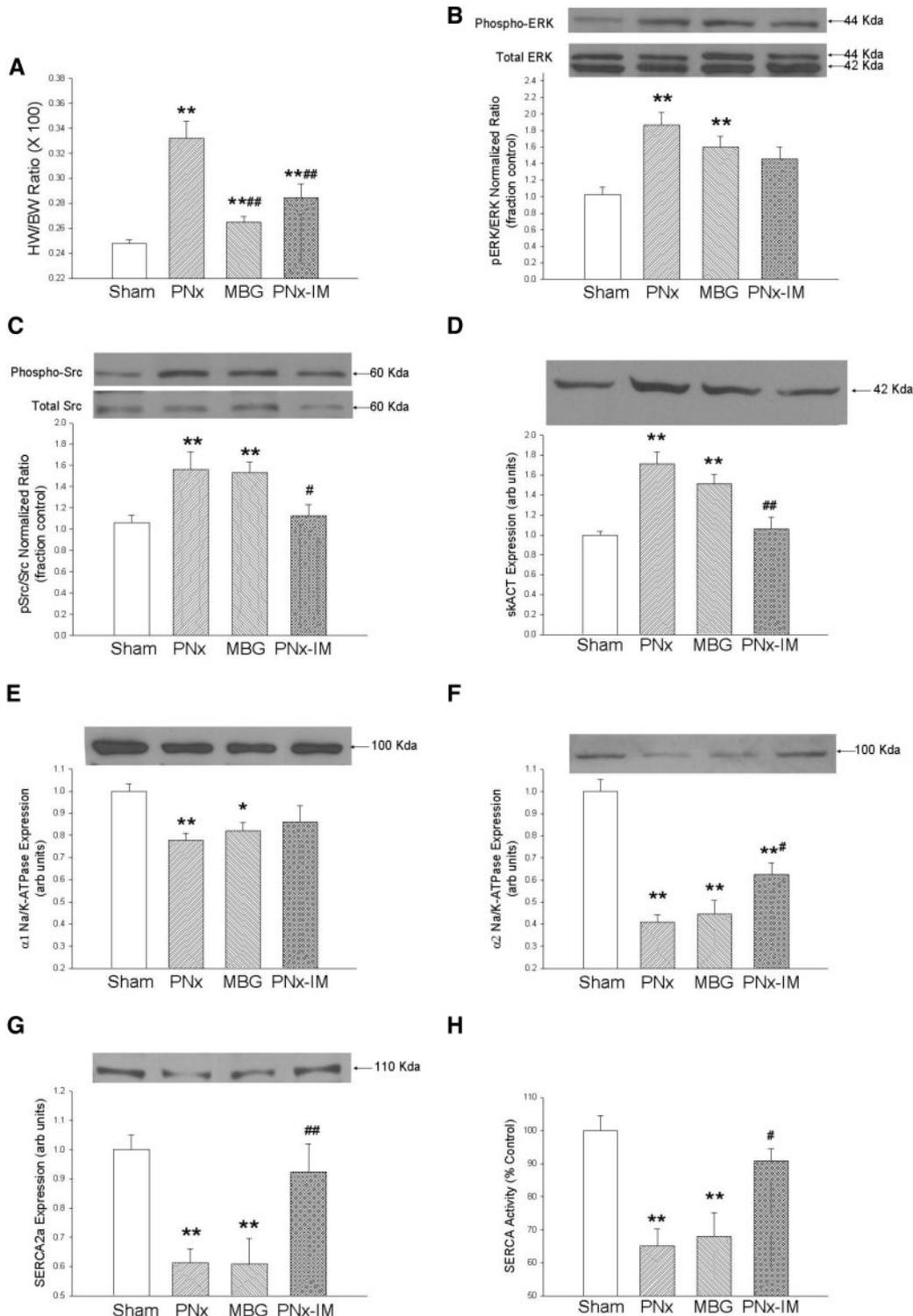
### Discussion

Patients with chronic renal failure develop cardiac disease with exceptional frequency.<sup>5</sup> Even mild degrees of chronic renal insufficiency are associated with marked increases in cardiovascular mortality.<sup>3</sup> 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.<sup>5</sup> The pathogenesis of this uremic cardiomyopathy has been debated.<sup>5,29,30</sup> Although 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.<sup>5</sup> It is interesting to note that patients with chronic renal failure also develop evidence for systemic inflammation and oxidant stress.<sup>26,27</sup> Oxidant stress is believed to be a major pathogenic factor in the cardiovascular disease seen in renal failure,<sup>31</sup> as well as the general population.<sup>32</sup>

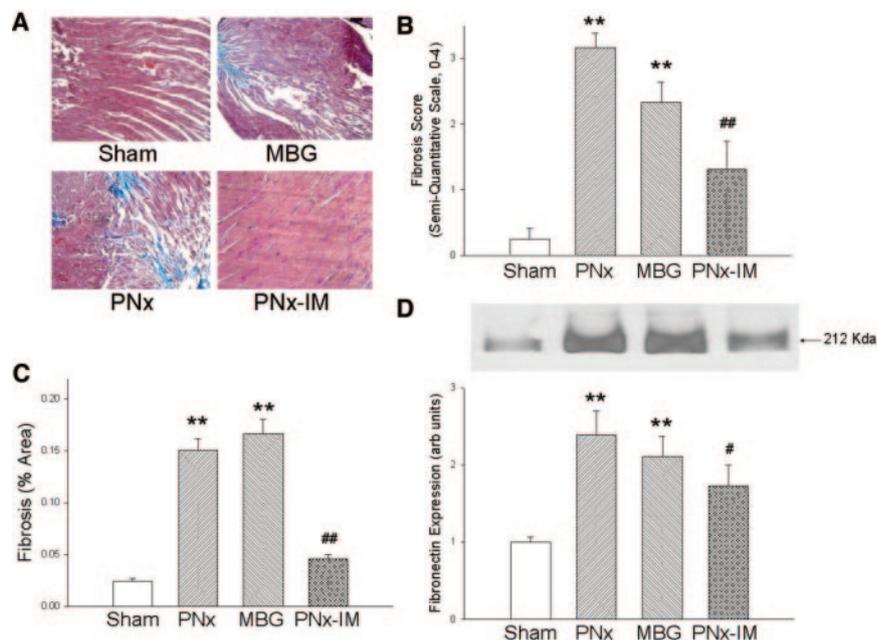
The concept that hormonal adaptations to decreases in renal function might participate in the pathogenesis of the uremic syndrome was elaborated in the 1960s. This concept is called "trade-off"; the idea is that body fluid and electrolyte homeostasis would be maintained despite renal insufficiency, but the elevated hormone levels might have deleterious consequences.<sup>12</sup> 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.<sup>33</sup> Interestingly, de Wardener,<sup>7</sup> Bricker,<sup>33</sup> 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. 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 that is mediated through Src, Ras, reactive oxygen species, and ERK and induce endocytosis of the plasmalemmal Na/K-ATPase.<sup>15-19</sup> This signal cascade occurs in cell-free systems and requires the Na/K-ATPase to be in caveolae to proceed.<sup>13,14</sup> Cardiotonic steroid signaling through the sodium pump causes well-described changes in gene expression, which can be blocked by antioxidants.<sup>11,18,19</sup>

The purpose of the current study was to examine whether this signaling by cardiotonic steroids through the Na/K-



**Figure 4.** MBG produces changes in cardiac morphology and protein expression consistent with experimental uremia. (a) Heart weight/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 before 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- $\mu$ g left ventricle homogenate protein. Representative active and total ERK blots shown. (c) Src (Src pY<sup>418</sup>) 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- $\mu$ g left ventricle homogenate protein. Representative active and total Src blots shown. (d) Skeletal muscle actin (skACT), (e) Na/K-ATPase  $\alpha 1$ , (f) Na/K-ATPase  $\alpha 2$ , and (g) SERCA2a expression 4 weeks after Sham ( $n=15$ ), PNx ( $n=13$ ), MBG ( $n=10$ ), or PNx-IM ( $n=6$ ). Gels for d through g were loaded with 20  $\mu$ g 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 ( $n=8$ ). 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 before partial nephrectomy (PNx-IM). (b) Semiquantitative 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  $\mu$ g of left ventricle homogenate protein. \* $P$ <0.05, \*\* $P$ <0.01 vs Sham, # $P$ <0.05, ## $P$ <0.01 vs PNx.



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.<sup>31,34</sup> This was not very surprising, because the rat partial nephrectomy model has been extensively studied as a model for chronic renal failure.<sup>4</sup> Next, we saw that infusion of 10  $\mu$ g/kg per 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 that 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,<sup>35</sup> and the markedly elevated levels of aldosterone have been implicated in this process.<sup>36</sup> 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 Randomized Aldactone Evaluation study.<sup>37</sup>

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,<sup>38</sup> and these settings are likely to have increased circulating concentrations of MBG.<sup>39,40</sup> 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 BP, 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 (eg, renin-angiotensin-aldosterone activation) in the pathogenesis of hypertension in our model.

Ferrandi et al<sup>41</sup> reported that longer-term infusions of ouabain (15  $\mu$ g/kg per day for 18 weeks) produced hypertension and cardiac hypertrophy in rats. 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.<sup>8</sup>

## Perspectives

Taken together, our data strongly support an important role for MBG in the pathogenesis of experimental uremic cardiomyop-

athy 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.

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### References

- Sarnak MJ, Levey AS, Schoolwerth AC, Coresh J, Culleton B, Hamm LL, McCullough PA, Kasiske BL, Kelepouris E, Klag MJ, Parfrey P, Pfeffer M, Raij L, Spinosa DJ, Wilson PW. Kidney disease as a risk factor for development of cardiovascular disease: a statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. *Circulation*. 2003;108:2154–2169.
- Garg AX, Clark WF, Haynes RB, House AA. Moderate renal insufficiency and the risk of cardiovascular mortality: results from the NHANES I. *Kidney Int*. 2002;61:1486–1494.
- Henry RM, Kostense PJ, Bos G, Dekker JM, Nijpels G, Heine RJ, Bouter LM, Stehouwer CD. Mild renal insufficiency is associated with increased cardiovascular mortality: The Hoorn Study. *Kidney Int*. 2002;62:1402–1407.
- Hostetter TH, Olson JL, Rennke HG, Venkatachalam MA, Brenner BM. Hyperfiltration in remnant nephrons: a potentially adverse response to renal ablation. *Am J Physiol*. 1981;241:F85–F93.
- Middleton RJ, Parfrey PS, Foley RN. Left ventricular hypertrophy in the renal patient. *J Am Soc Nephrol*. 2001;12:1079–1084.
- Kennedy D, Omran E, Periyasamy SM, Nadoor J, Priyadarshi A, Willey JC, Malhotra D, Xie Z, Shapiro JI. Effect of chronic renal failure on cardiac contractile function, calcium cycling, and gene expression of proteins important for calcium homeostasis in the rat. *J Am Soc Nephrol*. 2003;14:90–97.
- de Wardener HE. Franz Volhard Lecture 1996. Sodium transport inhibitors and hypertension. *J Hypertens*. 1996;14(suppl):S9–S18.
- Fedorova OV, Agalakova NI, Talan MI, Lakatta EG, Bagrov AY. Brain ouabain stimulates peripheral marinobufagenin via angiotensin II signaling in NaCl-loaded Dahl-S rats. *J Hypertens*. 2005;23:1515–1523.
- Fedorova OV, Talan MI, Agalakova NI, Lakatta EG, Bagrov AY. Endogenous ligand of alpha(1) sodium pump, marinobufagenin, is a novel mediator of sodium chloride-dependent hypertension. *Circulation*. 2002;105:1122–1127.
- Komiyama Y, Dong XH, Nishimura N, Masaki H, Yoshika M, Masuda M, Takahashi H. A novel endogenous digitalis, telocinobufagin, exhibits elevated plasma levels in patients with terminal renal failure. *Clin Biochem*. 2005;38:36–45.
- Priyadarshi S, Valentine B, Han C, Fedorova OV, Bagrov AY, Liu J, Periyasamy SM, Kennedy D, Malhotra D, Xie Z, Shapiro JI. Effect of green tea extract on cardiac hypertrophy following 5/6 nephrectomy in the rat. *Kidney Int*. 2003;63:1785–1790.
- Bricker NS. On the pathogenesis of the uremic state. An exposition of the "trade-off hypothesis". *N Engl J Med*. 1972;286:1093–1099.
- Liu J, Liang M, Liu L, Malhotra D, Xie Z, Shapiro JI. Ouabain-induced endocytosis of the plasmalemmal Na/K-ATPase in LLC-PK1 cells requires caveolin-1. *Kidney Int*. 2005;67:1844–1854.
- Liu L, Mohammadi K, Aynafshar B, Wang H, Li D, Liu J, Ivanov AV, Xie Z, Askari A. Role of caveolae in signal-transducing function of cardiac Na<sup>+</sup>/K<sup>+</sup>-ATPase. *Am J Physiol Cell Physiol*. 2003;284:C1550–C1560.
- Haas M, Askari A, Xie Z. Involvement of Src and epidermal growth factor receptor in the signal-transducing function of Na<sup>+</sup>/K<sup>+</sup>-ATPase. *J Biol Chem*. 2000;275:27832–27837.
- Huang L, Li H, Xie Z. Ouabain-induced hypertrophy in cultured cardiac myocytes is accompanied by changes in expression of several late response genes. *J Mol Cell Cardiol*. 1997;29:429–437.
- Kometiani P, Li J, Gnudi L, Kahn BB, Askari A, Xie Z. Multiple signal transduction pathways link Na<sup>+</sup>/K<sup>+</sup>-ATPase to growth-related genes in cardiac myocytes. The roles of Ras and mitogen-activated protein kinases. *J Biol Chem*. 1998;273:15249–15256.
- Liu J, Tian J, Haas M, Shapiro JI, Askari A, Xie Z. Ouabain interaction with cardiac Na<sup>+</sup>/K<sup>+</sup>-ATPase initiates signal cascades independent of changes in intracellular Na<sup>+</sup> and Ca<sup>2+</sup> concentrations. *J Biol Chem*. 2000;275:27838–27844.
- Xie Z, Kometiani P, Liu J, Li J, Shapiro JI, Askari A. Intracellular reactive oxygen species mediate the linkage of Na<sup>+</sup>/K<sup>+</sup>-ATPase to hypertrophy and its marker genes in cardiac myocytes. *J Biol Chem*. 1999;274:19323–19328.
- Periyasamy SM, Liu J, Tanta F, Kabak B, Wakefield B, Malhotra D, Kennedy DJ, Nadoor A, Fedorova OV, Gunning W, Xie Z, Bagrov AY, Shapiro JI. Salt loading induces redistribution of the plasmalemmal Na/K-ATPase in proximal tubule cells. *Kidney Int*. 2005;67:1868–1877.
- Bagrov AY, Roukoyatkina NI, Pinaev AG, Dmitrieva RI, Fedorova OV. Effects of two endogenous Na<sup>+</sup>,K<sup>(+)</sup>-ATPase inhibitors, marinobufagenin and ouabain, on isolated rat aorta. *Eur J Pharmacol*. 1995;274:151–158.
- Bagrov YY, Dmitrieva NI, Manusova NB, Zvartau EE, Patkina NA, Bagrov AY. Involvement of endogenous digitalis-like factors in voluntary selection of alcohol by rats. *Life Sci*. 1999;64:PL219–PL225.
- Bunag RD, Butterfield J. Tail-cuff blood pressure measurement without external preheating in awake rats. *Hypertension*. 1982;4:898–903.
- Langer SF. Differential laws of left ventricular isovolumic pressure fall. *Physiol Res*. 2002;51:1–15.
- Litwin SE, Katz SE, Morgan JP, Douglas PS. Serial echocardiographic assessment of left ventricular geometry and function after large myocardial infarction in the rat. *Circulation*. 1994;89:345–354.
- Himmelfarb J, McMonagle E, McMenamin E. Plasma protein thiol oxidation and carbonyl formation in chronic renal failure. *Kidney Int*. 2000;58:2571–2578.
- Himmelfarb J, McMonagle E. Albumin is the major plasma protein target of oxidant stress in uremia. *Kidney Int*. 2001;60:358–363.
- Simonides WS, van Hardeveld C. An assay for sarcoplasmic reticulum Ca<sup>2+</sup>(+)-ATPase activity in muscle homogenates. *Anal Biochem*. 1990;191:321–331.
- Rigatto C, Parfrey P, Foley R, Negrijn C, Tribula C, Jeffery J. Congestive heart failure in renal transplant recipients: risk factors, outcomes, and relationship with ischemic heart disease. *J Am Soc Nephrol*. 2002;13:1084–1090.
- Parfrey PS. Is renal insufficiency an atherogenic state? Reflections on prevalence, incidence, and risk. *Am J Kidney Dis*. 2001;37:154–156.
- Himmelfarb J, McMenamin E, McMonagle E. Plasma aminothiols oxidation in chronic hemodialysis patients. *Kidney Int*. 2002;61:705–716.
- Foley RN, Parfrey PS. Risk factors for cardiac morbidity and mortality in dialysis patients. *Curr Opin Nephrol Hypertens*. 1994;3:608–614.
- Bricker NS. The control of sodium excretion with normal and reduced nephron populations. The pre-eminence of third factor. *Am J Med*. 1967;43:313–321.
- Harnett JD, Kent GM, Barre PE, Taylor R, Parfrey PS. Risk factors for the development of left ventricular hypertrophy in a prospectively followed cohort of dialysis patients. *J Am Soc Nephrol*. 1994;4:1486–1490.
- Border WA, Noble NA. Transforming growth factor beta in tissue fibrosis. *N Engl J Med*. 1994;331:1286–1292.
- Greene EL, Kren S, Hostetter TH. Role of aldosterone in the remnant kidney model in the rat. *J Clin Invest*. 1996;98:1063–1068.
- Effectiveness of spironolactone added to an angiotensin-converting enzyme inhibitor and a loop diuretic for severe chronic congestive heart failure (the Randomized Aldactone Evaluation Study [RALES]). *Am J Cardiol*. 1996;78:902–907.
- Young MJ, Funder JW. Mineralocorticoid receptors and pathophysiological roles for aldosterone in the cardiovascular system. *J Hypertens*. 2002;20:1465–1468.
- Fedorova OV, Anderson DE, Bagrov AY. Plasma marinobufagenin-like and ouabain-like immunoreactivity in adrenocorticotropin-treated rats. *Am J Hypertens*. 1998;11:796–802.
- Fedorova OV, Doris PA, Bagrov AY. Endogenous marinobufagenin-like factor in acute plasma volume expansion. *Clin Exp Hypertens*. 1998;20:581–591.
- Ferrandi M, Molinari I, Barassi P, Minotti E, Bianchi G, Ferrari P. Organ hypertrophic signaling within caveolae membrane subdomains triggered by ouabain and antagonized by PST 2238. *J Biol Chem*. 2004;279:33306–33314.

## Central Role for the Cardiotonic Steroid Marinobufagenin in the Pathogenesis of Experimental Uremic Cardiomyopathy

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