Renal Ischemia Regulates Marinobufagenin Release in Humans

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Abstract—Cardiotonic steroids, including marinobufagenin, are a group of new steroid hormones found in plasma and urine of patients with congestive heart failure, myocardial infarction, and chronic renal failure. In animal studies, partial nephrectomy induces marinobufagenin elevation, cardiac hypertrophy, and fibrosis. The objective of this study is to test the effect of renal ischemia on marinobufagenin levels in humans with renal artery stenosis (RAS). To test this, plasma marinobufagenin levels were measured in patients with RAS of the Prospective Randomized Study Comparing Renal Artery Stenting With or Without Distal Protection, non-RAS patient controls who were scheduled for coronary angiography, and normal healthy individuals. Marinobufagenin levels were significantly higher in patients with RAS compared with those of the other 2 groups. Multivariate analysis shows that occurrence of RAS is independently related to marinobufagenin levels. In addition, renal artery revascularization by stenting partially reversed marinobufagenin levels in the patients with RAS (0.77±0.06 nmol/L at baseline; 0.66±0.06 nmol/L at 24 hours; and 0.61±0.05 nmol/L at 1 month). In conclusion, we have found that marinobufagenin levels are increased in patients with RAS, whereas reversal of renal ischemia by stenting treatment reduces marinobufagenin levels. These results suggest that RAS-induced renal ischemia may be a major cause of marinobufagenin release. **(*Hypertension*. 2010;56:00-00.)**

Key Words: renal artery stenosis ■ hypertension ■ cardiotonic steroids ■ marinobufagenin ■ renal artery stenting

Cardiotonic steroids (CTSs) are a group of steroid hormones that have been found recently in mammals including humans.[1] There is evidence demonstrating that these compounds can be synthesized endogenously and possess identical structures as their plant- and amphibian-originated counterparts.[2,3] In humans, an endogenous CTS, marinobufagenin (MBG), was isolated and identified from the urine of myocardial infarction patients[4] and from uremic plasma.[2] Both in vitro and in vivo studies demonstrated that epinephrine, angiotensin II, and adrenocorticotropic hormone could induce adrenal cortical cells to release endogenous CTS.[5–7] Other factors that can stimulate CTS include physical exercise,[8] hypoxia,[9] and behavioral stress.[10]

Renal artery stenosis (RAS) is a major cause for secondary hypertension in the United States.[11] Importantly, RAS-induced hypertension has a 3 times higher incidence of adverse cardiovascular (CV) events than those with essential hypertension when matched with equivalent blood pressure.[12–14] It has been reported that CTS levels increase in patients and animals with volume-expanded hypertension or preeclampsia,[15,16] as well as renal failure.[2] We have recently demonstrated in animal models that partial nephrectomy increases plasma MBG levels and induces hypertension and cardiac fibrosis.[17] Neutralization of MBG by active immunization against an MBG-albumin conjugate attenuates the pathological cardiac fibrosis in rats.[18,19] The objective of the current study was to test whether renal ischemia induced by RAS alters MBG levels in humans.

Methods

Subjects

All of the subjects provided written informed consent from a protocol approved by an institutional review board. RAS subjects were from the Prospective Randomized Study Comparing Renal Artery Stenting With or Without Distal Protection (RESIST), which was conducted by the University of Toledo Clinical Coordinating Center.[20] The inclusion criteria were patients with hypertension and ≥1 RAS of ≥50% and <100%, treatable with stenting. The primary exclusion criteria were a systolic blood pressure >200 mm Hg or diastolic blood pressure >120 mm Hg on the day of randomization, age <18 years, pregnancy, dialysis, kidney transplant, kidney size <8 cm, restenosis, or stroke, major surgery, congestive heart failure, major trauma, and myocardial infarction within a short period of time of planned enrollment. All of the patients successfully received stenting treatment. Patient control subjects were adult patients who have a history of hypertension or angina scheduled for coronary angiography and no RAS. Normal healthy control subjects were healthy individuals (age >18 years) who have no history of hypertension, angina, or RAS.

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Blood Sample Collection

All of the peripheral venous blood samples were collected in lithium heparin plasma separator tubes, spun at 1000*g* for 15 minutes, and the obtained plasma samples were stored at −80°C until analysis. RAS patients also had samples collected at 24 hours and 1 month poststenting during the RESIST.

Measurement of Plasma MBG

The plasma sample extraction was based on the method described before.[21] The disposable Sep-Pak C-18 columns (Waters) were activated by 10 mL of 100% acetonitrile and washed once with 5 mL of distilled water. Plasma samples (0.5 mL) were then loaded onto the columns. After another washing step with 5 mL of distilled water, each column was eluted with 7 mL of 20% acetonitrile followed by 7 mL of 80% acetonitrile. The eluates were combined, lyophilized, and resuspended in Tris-buffered saline buffer (50 mmol/L of Trizma, 150.0 mmol/L of NaCl, and 7.7 mmol/L of NaN_3 [pH 7.4]). The concentration of MBG was then determined using a competitive ELISA based on a 4G4 anti-MBG murine monoclonal antibody.[22] Briefly, 100 μL of MBG standards or sample eluates were mixed with 100 μL of anti-MBG monoclonal antibody (1:1000 dilution in Tris-buffered saline buffer with 1.00% BSA and 0.25% Tween 20). The mixture was then added to a MBG-thyroglobulin–coated and 1% BSA-blocked ELISA plate. After 1 hour of incubation, plates were washed 3 times, and secondary antimouse antibody conjugated with alkaline phosphatase (1:10 000 dilution in Tris-buffered saline buffer with 1.00% BSA and 0.25% Tween 20) was added and incubated for another 1 hour. A fluorescent signal amplifier, FDP (fluorescein diphosphate, tetraammonium salt), the substrate of alkaline phosphatase from ANASpec, was used to detect the signals after washing out the secondary antibody. The sample MBG concentrations were calculated based on the standard curve using the MBG compound. MBG was purified from parotid secretion of a Bufo marinus toad, and MBG-thyroglobulin was synthesized as reported previously.[23] The secondary antimouse antibody was purchased from Sigma.

Other Laboratory Analyses

The baseline biomarkers were measured as described before.[20] Glomerular filtration rate (GFR) was calculated from the modified Modification of Diet in Renal Disease equation[20,24] and was used as the primary measure of renal function.

Statistical Analysis

Study data are presented as continuous (mean±SE) and categorical data. Because the MBG data are not normally distributed in RAS patients, the Kruskal-Wallis test was used for the analysis to detect the significance among the RAS patients and the control subjects. Repeated-measure ANOVA was used to test the changes among the baseline, 24-hour, and 1-month poststenting for the RAS patients. We also performed a Fisher least significant difference procedure to detect the pairwise difference. Multivariate analysis was conducted using linear logistic regression. All of the analyses were performed with SAS 9.1 or JMP software (SAS Inc).

Results

RAS-Induced Renal Ischemia Increases Plasma MBG Levels

To test whether RAS-induced renal ischemia increases plasma MBG levels, we first compared the plasma MBG concentration in RAS patients with that in normal healthy individuals (age: 30 to 55 years and no hypertension, angina, or RAS history). The result demonstrated that MBG levels are significantly higher in RAS patients (0.77±0.06 nmol/L, n=49, versus 0.25±0.02 nmol/L, n=26, in control subjects; $P<0.01$; Figure 1). Because MBG levels were also found elevated in patients with myocardial infarction and hypertension,[4,15] we then compared 60 non-RAS hypertensive patients who were scheduled for coronary angiography as an additional control group to test whether renal ischemia specifically contributes to the increased MBG in RAS patients. To eliminate the confounding factors, we compared the basic characteristics of the RAS and non-RAS patients, including their age, sex, medical history, blood pressure, kidney function, medications, and other risk factors. As shown in Table 1, the MBG concentration is significantly higher in RAS patients than in non-RAS patient controls (0.77±0.06 versus 0.20±0.06 nmol/L; $P<0.01$). Other basic characteristics of the RAS and non-RAS patients were listed in Table 1. Among these variables, the age, systolic blood pressure, presence of hypertension, GFR, use of diuretics, and use of angiotensin-converting enzyme inhibitor (ACEi)/angiotensin II receptor blocker (ARB) were significantly different between the 2 groups, and serum creatinine was close to significance. To test whether these factors are independently associated with the MBG elevation, we performed the multivariate analysis. All of the variables in Table 1 with $P\leq0.1$ were included in the multivariate analysis. As shown in Table 2, the occurrence of RAS and use of ACEi/ARB are independently associated with the increased plasma MBG levels in the multivariate model. For patients with RAS, the plasma MBG levels are significantly higher in patients receiving ACEi/ARB treatment than in patients without receiving ACEi/ARB treatment (0.93±0.08 nmol/L, n=26, versus 0.63±0.08

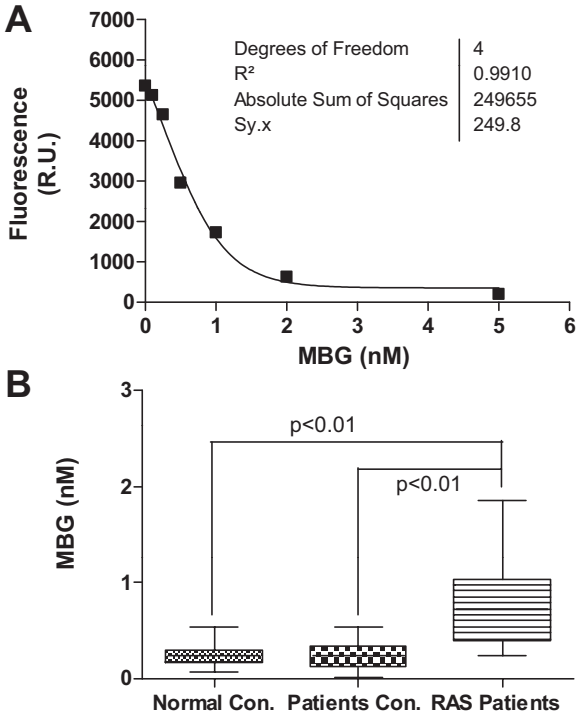

Figure 1. Plasma MBG levels in RAS patients and control subjects. The MBG concentration was measured in plasma samples from RAS patients or control subjects as described in the Methods section. A, Representative standard curve of MBG measurement using purified MBG compound; B, distribution and mean MBG levels in normal healthy controls (Normal con.), hypertensive patient controls (Patients con.), and patients with RAS (RAS patients).

Table 1. Basic Characteristics of RAS Patients and Non-RAS Patient Controls

Variables	Non-RAS Patients, (n=60)	RAS Patients, (n=49)	*P*, Non-RAS vs RAS
Plasma levels of CTS			
MBG, nM	0.20±0.06	0.77±0.06	<0.01
Demographic characteristics			
Age, y	61.4±1.5	70.5±1.3	<0.01
Women, n (%)	28 (46)	30 (61)	0.18
White, n (%)	52 (87)	45 (92)	0.54
BMI	31.1±1.1	28.9±0.8	0.10
Systolic BP, mm Hg	131±2	159±5	<0.01
Diastolic BP, mm Hg	77±1	76±2	0.57
Heart rate, bpm	68±1	67±2	0.63
Indications for treatment, n (%)			
Hypertension	43 (72)	48 (98)	<0.01
Angina	24 (40)	23 (47)	0.56
Laboratory values			
Serum creatinine, mg/dL	0.98±0.03	1.11±0.06	0.07
MDRD GFR	78.3±4.4	64.7±3.7	0.02
Risk factors, n (%)			
CAD	32 (53)	34 (69)	0.08
Diabetes mellitus	15 (25)	11 (22)	0.82
History of smoking	31 (52)	34 (69)	0.08
Medications, n (%)			
ACEi/ARB	18 (31)	23 (47)	0.08
Diuretics	14 (24)	23 (47)	0.02

BMI, body mass index; BP, blood pressure; CAD, coronary artery disease; MDRD GFR, GFR using modification of diet in renal disease. Values are mean±SE or No. (%) of patients.

nmol/L, n=23; $P<0.05$). Figure 2 shows that MBG levels in patient plasma samples are correlated with the severity of the RAS. The average MBG concentrations are 0.20±0.06 nmol/L in patients without RAS, 0.69±0.07 nmol/L in patients with unilateral RAS (n=32), and 0.88±0.12 nmol/L in patients with bilateral RAS (n=16), respectively.

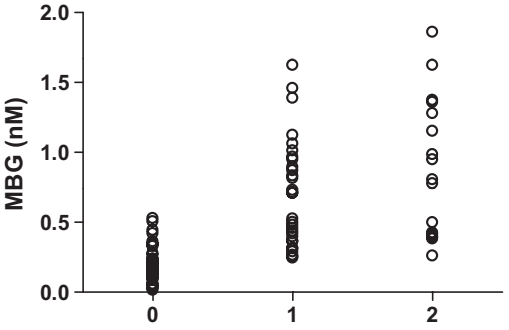

Figure 2. Distribution of plasma MBG levels in patients categorized by the severity of RAS (0 indicates patients without RAS; 1, patient with unilateral RAS; 2, patients with bilateral RAS or RAS patients with only a solitary kidney). The average MBG concentrations are 0.20±0.06 nmol/L in patients without RAS, 0.69±0.07 nmol/L in patients with unilateral RAS (n=32), and 0.88±0.12 nmol/L in patients with bilateral RAS (n=16), respectively.

Reversal of Renal Ischemia by Stenting Reduces MBG Levels

To further confirm that renal ischemia is a cause of MBG elevation in RAS patients, we measured the plasma MBG levels of RAS patients at 24 hours and 1 month after the stenting. A total of 49 available paired samples were tested. The result demonstrated that MBG levels decreased after stenting (0.77±0.06 nmol/L baseline versus 0.66±0.05 nmol/L at 24 hours and 0.60±0.05 nmol/L at 1 month; $P<0.05$; Figure 3). MBG levels at 24 hours and 1 month were significantly lower than the baseline levels, but no further reduction was seen from 24 hours to 1 month. Because the RESIST patients were randomized into 4 groups (control group, Angioguard-only group, abciximab-only group, and Angioguard plus abciximab group) before receiving the renal artery stenting treatment, we also compared the changes of MBG and found no significant differences between these groups (Figure 4).

To test whether MBG levels were related to renal function, we analyzed the creatinine concentrations in plasma samples

Table 2. Multivariate Analysis for Predictors of Plasma MBG Levels

Model	Unstandardized Coefficients		Standardized Coefficients, β	t	Sig.	95% CI for B	
	B	SE				Lower Bound	Upper Bound
Constant	0.364	0.447		0.814	0.418	−0.524	1.252
Age	3.667×10^{-5}	0.003	0.001	0.012	0.990	−0.006	0.006
BMI	−0.005	0.005	−0.077	−0.950	0.345	−0.015	0.005
Serum creatinine	−0.214	0.150	−0.188	−1.424	0.158	−0.512	0.085
MDRD GFR	0.000	0.002	−0.024	−0.182	0.856	−0.004	0.003
Systolic BP	0.001	0.001	0.103	1.262	0.210	−0.001	0.004
RAS_severity	0.348	0.047	0.633	7.478	0.000	0.255	0.440
ACEi/ARB	0.152	0.065	0.178	2.351	0.021	0.024	0.281
Diuretics	0.009	0.066	0.010	0.136	0.892	−0.122	0.140

Dependent variable is Baseline_MBG. BMI indicates body mass index; systolic BP, systolic blood pressure; RAS, renal artery stenosis; MDRD GFR, GFR using modification of diet in renal disease; Sig., significance.

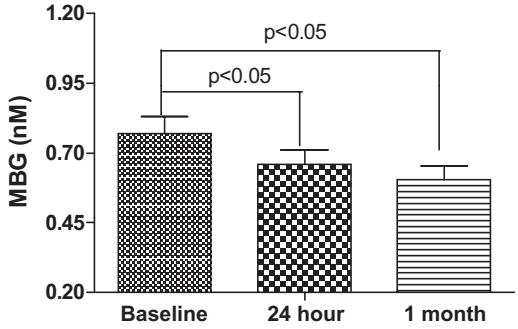

Figure 3. MBG levels at baseline, 24 hours, and 1 month after renal artery stenting in RAS patients. The MBG levels were measured in the plasma samples collected from patients at 3 time points. The baseline sample was collected immediately before the patient receiving the renal artery stenting, and the poststenting samples were collected at 24 hours and 1 month after receiving the renal artery stenting, respectively.

and calculated the GFR from RAS patients at baseline, 24 hours poststenting, and 1 month poststenting. As shown in Figure 5, MBG changes after stenting correlated with the GFR changes in patients with bilateral RAS (r=0.57; P<0.05) but not in patients with unilateral RAS (r=0.12; P>0.05).

Discussion

In the current study we observed that patients with RAS appear to have elevated plasma levels of MBG when contrasted against either healthy adults or a comparator group of hypertensive patients (Figure 1 and Table 1). MBG is a bufadienolide type of CTS originally found from parotid secretion of the Bufo marinus toad.[23] It has been demonstrated that MBG can also be synthesized in animal adrenal glands[25] and in cultured adrenocortical cells.[26] Like other CTSs, MBG inhibits Na/K-ATPase activity and may potentially regulate the sodium reabsorption and kidney function in conditions of high-salt loading or plasma volume expansion.[27,28] Increased levels of CTS, including MBG, have been reported in patients with hypertension, myocardial infarction, and heart failure,[4,29,30] as well as in patients with end-stage renal disease and chronic renal diseases.[2,31] There is evidence from the current study showing that renal ischemia induced by RAS is causally related to increased plasma MBG in these patients. First, we found a good correlation between the severity of RAS and the plasma MBG levels in the RAS patients, as shown in Figure 2. Importantly, reversal of renal ischemia by stenting reduced the plasma MBG levels in these patients. Finally, these results are consistent with our previous animal experiments. For example, in partial nephrectomy-induced renal ischemia animals and in salt-loaded animals, both the plasma MBG level and the urine MBG excretion increased.[17,19,32]

The mechanism(s) linking renal ischemia to increased concentration of MBG in humans has not been characterized. Because the MBG was found mainly synthesized in the adrenal gland,[25] renal ischemia may trigger the release of hormones from the kidney or pituitary that, in turn, stimulates

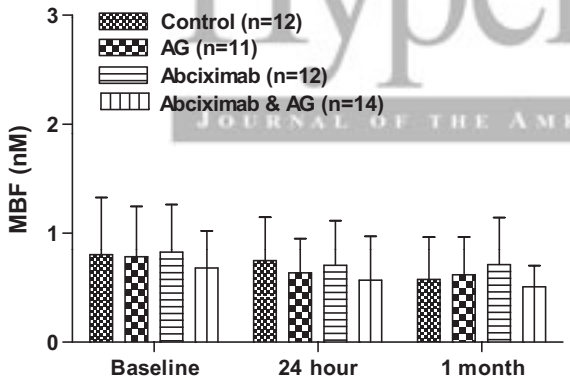

Figure 4. MBG levels in the randomized groups of RESIST at baseline, 24 hours, and 1 month. RAS patients were randomly assigned into 4 groups before receiving the renal artery stenting, as described previously.[20] The control group received neither Angioguard nor abciximab); the Angioguard-only group (AG) received Angioguard during the stenting procedure; the abciximab-only group (Abciximab) received abciximab treatment before the stenting procedure; and the Angioguard and abciximab group (Abciximab & AG) received both abciximab treatment before stenting and the Angioguard during the stenting procedure. The plasma MBG concentrations were analyzed in the above 4 groups at their baseline and 24 hours and 1 month poststenting.

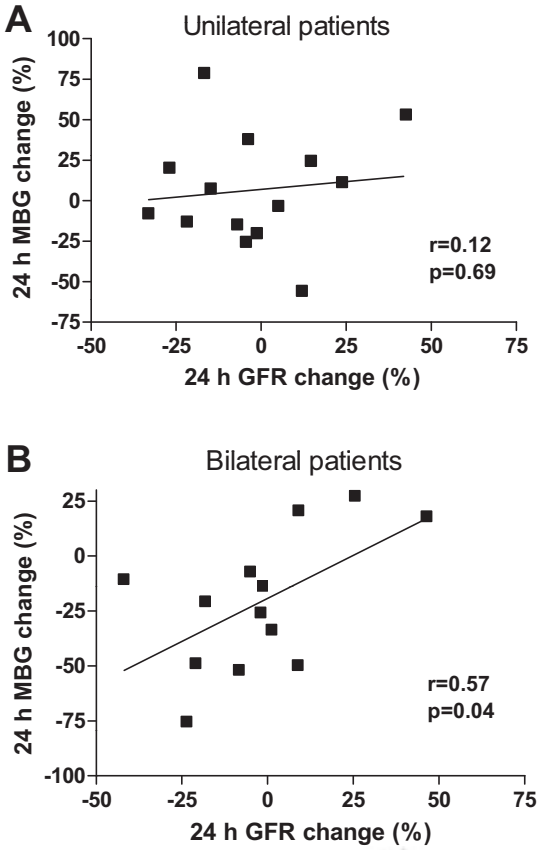

Figure 5. Correlation between MBG and GFR changes in RAS patients after renal artery stenting. The percentage changes of MBG and GFR were calculated using the following formula: 24 hour change (%)=(24 hour value−baseline value)/baseline value×100%. Negative values indicate a decrease in MBG or GFR after stenting. A, Correlation of 24-hour change between MBG and GFR in patients with unilateral RAS; B, correlation of 24-hour change between MBG and GFR in patients with bilateral RAS.

the release of MBG from the adrenal gland. Specifically, elevation of angiotensin II has been reported to regulate CTS release from the adrenal cortex.[6,8,27] Despite this, the current study found that RAS patients on ACEi/ARB treatment had higher plasma MBG levels compared with the patients without ACEi/ARB treatment. It is not known whether reduced GFR in these patients has any effects on the MBG excretion, which may merit further studies to measure the 24-hour urine MBG excretion in the RAS patients.

On the other hand, MBG has been found to have a natriuretic effect in animal models with salt-induced volume expansion.[32,33] MBG, as well as other CTS compounds, can induce the protein endocytosis of the kidney proximal tubule Na/K-ATPase.[34,35] Reduced Na/K-ATPase protein and activity on the basolateral membrane of kidney proximal tubules blunt the sodium reabsorption and, therefore, increase natriuresis. The current study has not shown an independent association between the renal function (plasma creatinine level or GFR) and the baseline MBG levels. However, as shown in Figure 5, the reduction of MBG at 24 hours after renal artery stenting correlated with a decrease in GFR in patients with bilateral RAS but not in patients with unilateral RAS. We hypothesize that an acute reduction of MBG may affect the kidney function in these patients. However, it requires further study to explain the mechanisms involved in this effect.

Elevated levels of MBG may help explain the relationship between RAS and CV events. Wollenweber et al[36] described a 6-year CV event-free survival of 53%, with risk related to the severity of the renal stenosis. Several others have suggested that the risk of adverse CV events is high and occurs in excess of the hypertension severity.[37–39] More recently, a significant difference in 4-year survival was seen between those with incidental RAS compared with those without, with a graded effect on mortality, according to the severity of RAS.[40] In RAS patients specifically, renal dysfunction is associated with increased CV event rates and increased mortality.[41,42] Ventricular dysfunction and overt congestive heart failure are common in patients with RAS, just as RAS is common in patients with congestive heart failure.[43] The elevation of endogenous CTS has now been linked with a variety of CV and renal disease settings.[44–49] Animal experiments using rats and mice have demonstrated that renal ischemia induced by partial nephrectomy increases MBG and causes diastolic dysfunction and cardiac fibrosis.[17–19] Importantly, the cardiac fibrosis seen in such animals can be prevented by immunization against MBG, whereas infusion of MBG results in a similar pathological lesion. Our result of MBG elevation may indicate that MBG is an important attributor to the increased CV events in RAS patients.

Perspectives

The current study shows that renal ischemia is associated with high levels of a circulating endogenous CTS, MBG. Recent work in this area demonstrates that CTSs are likely important intermediaries in the linkage between chronic kidney disease and the development of cardiac hypertrophy and fibrosis. Thus, the MBG elevation may in part attribute to the high CV events in patients with RAS, and the measurement of plasma MBG levels may serve as a biomarker for the cardiorenal syndrome.

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Disclosures

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