

# Effects of Resibufogenin in Experimental Hypertension

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## Key Words

Marinobufagenin · Experimental hypertension · Volume expansion · Angiotensinogen · Resibufogenin

## Abstract

**Background/Aims:** There are two major pathophysiologic processes involved in the development of hypertension: (1) expanded extracellular fluid volume and (2) vasoconstriction. We have developed a model of preeclampsia in the rat, in which excessive volume expansion (VE) plays a role. These animals excrete increased amounts of the bufodienolide, marinobufagenin (MBG), even before their hypertension and proteinuria become established. Furthermore, their hypertension is corrected by administration of resibufogenin (RBG), a compound structurally similar to MBG. **Method:** We studied two models of experimental hypertension in the nonpregnant animal, produced either by deoxycorticosterone acetate (DOCA)-salt administration or by angiotensin infusion. **Results:** RBG administered to the DOCA-salt rats lowered blood pressure and reduced proteinuria in the VE animals, but had no effect on the rats infused with angiotensin. Furthermore, although the production of superoxide anion in the aortas of both groups of hypertensive rats was increased over control, RBG reduced these levels to normal in the VE (DOCA-salt) animals only. RBG had no effect in the angiotensin-infused rats. The urinary excretion of angiotensinogen did not rise in VE-mediated hypertension, but did

increase in the angiotensin-infused rats. **Conclusions:** MBG plays an important role in the causation of hypertension in the VE rats, but not in the vasoconstrictive model. RBG is effective only in VE-mediated hypertension.

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## Introduction

The model of administration of salt and the powerful mineralocorticoid deoxycorticosterone acetate (DOCA) following uninephrectomy has been utilized to simulate 'low renin' or 'volume expansion (VE) mediated' hypertension [1, 2]. While DOCA-salt has additional influences on sodium handling (e.g., an increase in renal nerve activity) [3], the final effect on blood pressure (BP) remains its actions to produce VE [4]. We have employed this model in the nonpregnant rat to compare and contrast its pathophysiology with that involved in a vasoconstrictive form of experimental hypertension caused by the infusion of angiotensin II [5].

In work previously reported from this laboratory [6], we have employed a model of VE-mediated hypertension to study the syndrome of human preeclampsia in the rat. We determined that the excretion of a circulating inhibitor of Na<sup>+</sup>/K<sup>+</sup>-ATPase, marinobufagenin (MBG), is elevated in this model *prior* to the advent of hypertension and proteinuria [7]. MBG is a cardenolide of the bufadi-

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enolide class [8], compounds of which have vasoconstrictive properties [9]. Furthermore, we have determined that a congener of MBG, resibufogenin (RBG), is an antagonist of MBG [10]. However, although it is clear that both RBG and MBG inhibit  $\text{Na}^+/\text{K}^+$ -ATPase [10], the mechanism by which RBG serves as an antagonist to MBG is not clear. Thus, rather than preventing an alteration in the activity of MBG on the enzyme, the antagonism may result from an effect on other cellular/signaling mechanisms. Studies to elucidate these mechanisms are currently under way.

With these previous studies as background, we attempted to determine whether RBG would be effective in another model of VE-mediated hypertension in the non-pregnant animal. According to our thesis, if VE-mediated hypertension involves the excessive elaboration of MBG, whereas that due to angiotensin excess does not, then RBG should lower the BP in rats made hypertensive with the administration of DOCA and salt, whereas it should not alter the hypertension due to angiotensin infusion. This report details the results of experiments designed to test this hypothesis.

## Materials and Methods

Male CD rats (300–350 g; Charles River Laboratories, Wilmington, Mass., USA) were housed on a 12-hour light/12-hour dark schedule and allowed free access to standard rat chow (Laboratory Rodent Diet 5001; Purina Mills, St. Louis, Mo., USA) and tap water. The animals were randomly divided into five experimental groups: (1) NDS animals ( $n = 5$ ). Left nephrectomy was performed under ketamine anesthesia (0.8 mg/kg i.p.), and the rats were allowed to recover for 1 week. They were then injected with DOCA suspended in oil intraperitoneally, with an initial dose of 12.5 mg followed by a 6.5-mg injection weekly. After the animals had become hypertensive, the study was continued for a total of 2 weeks. (2) NDSR animals ( $n = 10$ ). In an additional 10 NDS rats, RBG was administered intraperitoneally on a daily basis at a dose of 30  $\mu\text{g}/\text{kg}$  once hypertension was established (at about 5 days). The drug was then continued for another 2 weeks. (3) SHM rats. Six rats were sham operated and then allowed to recover with no additional experimental maneuvers. These animals received tap water as drinking water. (4) ANG rats. In 5 animals, angiotensin II (40–50  $\mu\text{g}/\text{min}$ ) was infused via an osmotic minipump (model 2002; ALZET Osmotic Pumps, Cupertino, Calif., USA) which was implanted subcutaneously in the scapular region at the dorsum of the neck for 13 days. (5) ANGR rats. In 10 angiotensin-infused rats, RBG at the dose noted above was injected after 5 days, when hypertension had been established, for an additional 8 days.

The Tulane University Animal Care and Use Committee approved the experimental procedures, and all animal studies were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

## BP, Protein Excretion, Creatinine, and Hematocrit Measurements

The systolic BP was measured by the tail cuff method (model 59 system; IITC Life Science, Woodland Hills, Calif., USA). For each BP value reported, on average three to five readings were performed, when the BP had stabilized. The measurements were obtained at four different time points:  $t_0$  = before any treatment was started;  $t_1$  = when hypertension was established;  $t_2$  = on the 3rd to 5th day after RBG injection was begun, and  $t_3$  = on the day of sacrifice.

The 24-hour urinary protein excretion was measured using the micro pyrogallol red method (total protein kit; Sigma-Aldrich, St. Louis, Mo., USA). A Creatinine Analyzer 2 and the creatinine reagent kit (picric acid method; Beckman Coulter, Fullerton, Calif., USA) were used for creatinine determinations in blood and urine. The hematocrit was measured using an Autocrit Ultra-3 direct-reading centrifuge (BD Diagnostic Systems, Sparks, Md., USA).

## Evaluation of Oxidative Stress

To evaluate oxidative stress in the rat aorta, reactive oxygen species (ROS)-sensitive luminescent dye L-012 (Wako Pure Chemical Industries, Osaka, Japan) was used. Specificity and linearity of ROS detection by L-012 were verified in vitro using the superoxide donor paraquat (0–5.5 mmol/l) or  $\text{H}_2\text{O}_2$  (0–0.5 mmol/l) with/without antioxidant (Tiron, 1 mmol/l; Sigma-Aldrich). We obtained a dose-dependent linear correlation between the L-012 luminescent signal and the concentration of paraquat or  $\text{H}_2\text{O}_2$  and complete signal elimination when antioxidant was present (data not shown).

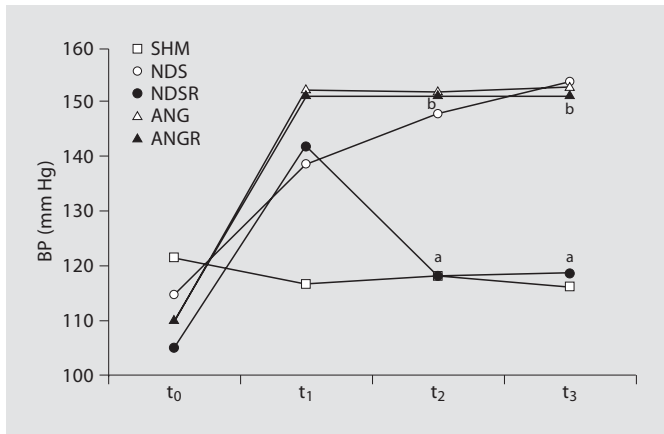
To measure ROS, the whole aorta was dissected, cleaned from adherent fat and connective tissue, washed twice in Krebs-HEPES buffer (pH 7.4, containing in mmol/l: NaCl 100, KCl 5,  $\text{CaCl}_2$  2,  $\text{MgSO}_4$  1.2,  $\text{K}_2\text{HPO}_4$  1, Na-HEPES 25, and glucose 11), and cut into 2- to 3-mm segments. Aortic segments were placed in pre-weighed test tubes with 100  $\mu\text{mol}/\text{l}$  L-012 in the same buffer (0.5 ml) and incubated in the dark for 30 min at 37°C. The luminescence signal was measured with a TD-20/20 luminometer (collection time 1 min; Turner Designs, Sunnyvale, Calif., USA), normalized for dry weight, and expressed as relative light units (RLU) per minute per dry aortic tissue weight.

## Measurements of Urinary Angiotensinogen

The urinary excretion of angiotensinogen was evaluated by angiotensin I ELISA, as previously reported [11–13]. ELISA was performed to measure the angiotensin I generated following incubation of samples with excess amounts of human renin (60  $\mu\text{U}$ ; Sigma-Aldrich). ELISA of angiotensin I was performed with a commercially available kit (Phoenix Pharmaceuticals, Burlingame, Calif., USA). Urine samples were incubated with renin for 60 min at 37°C in 100 mmol/l phosphate buffer (pH 6.5) containing 10 mmol/l DFP and 10 mmol/l EDTA-2Na. Conversion of all angiotensinogen to angiotensin I was observed at 60 min. From the maximum concentration of angiotensin I, the concentration of angiotensinogen was calculated under the assumption that 1 mol of angiotensin I was produced from 1 mol of angiotensinogen [13].

## Statistics

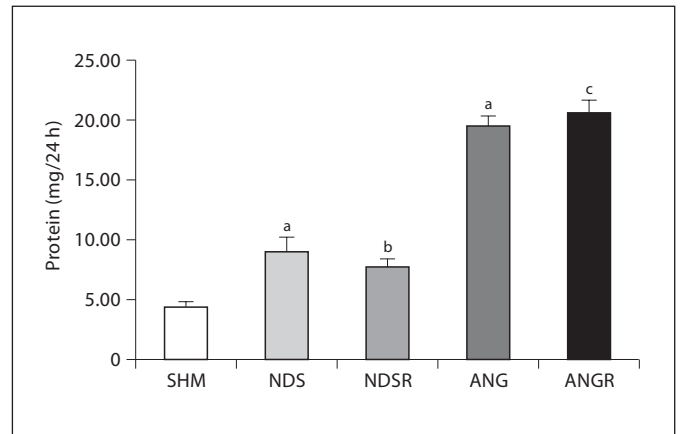
Values are presented as mean  $\pm$  SEM. Statistical comparison for multiple determinations was performed using a one-way ANOVA with Tukey's post hoc test.  $p < 0.05$  was considered significant.



**Fig. 1.** Mean systolic BP values in SHM animals, ANG rats, ANGR animals, NDS rats, and NDSR animals. Time intervals: t<sub>0</sub> = baseline; t<sub>1</sub> = following 5 days of angiotensin II infusion in ANG and ANGR groups and after 10 days of saline and DOCA administration in the NDS and NDSR rats; t<sub>2</sub> = 3–5 days after institution of RBG in the ANGR and NDSR groups, and t<sub>3</sub> = after 12–14 days of treatment with RBG in the NDSR group and after 7–8 days of RBG treatment in the ANGR group. RBG was effective in lowering BP to normal in the NDSR group of rats (<sup>a</sup> p < 0.05 vs. NDS), but had no effect on the BP in ANGR rats (<sup>b</sup> p > 0.05 vs. ANG).

## Results

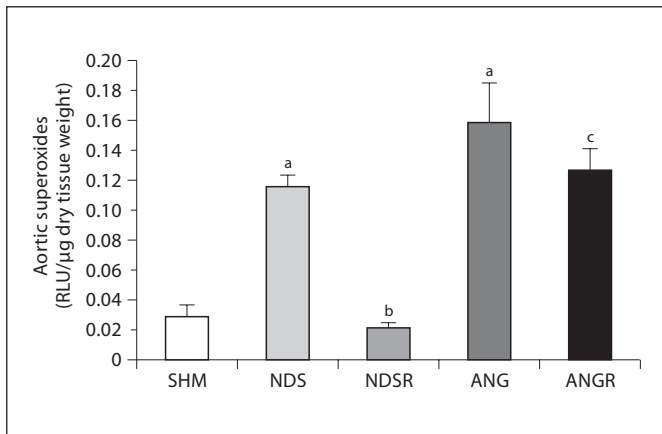
The BP rose in the angiotensin-infused group (ANG group) from levels averaging  $110 \pm 1$  to  $152 \pm 1$  mm Hg after 5 days (t<sub>1</sub>, fig. 1, p < 0.001) and remained elevated throughout the study. The administration of RBG (ANGR group) did not affect these BP values. The mean BP at t<sub>3</sub> (1 week after daily RBG administration was begun) was virtually identical in the ANG and ANGR groups (ANG group:  $153 \pm 1$  mm Hg, ANGR group:  $152 \pm 1$  mm Hg; p > 0.05). The animals subjected to uninephrectomy and administration of DOCA and saline (NDS animals) had an increase in BP from  $115 \pm 4$  to  $139 \pm 2$  mm Hg after 10 days (p < 0.001), to  $148 \pm 5$  mm Hg at t<sub>2</sub> (p < 0.001 vs. t<sub>0</sub>), and to  $154 \pm 3$  mm Hg at t<sub>3</sub> (p < 0.001 vs. t<sub>0</sub>). The NDS group treated with RBG (NDSR group) showed a decline in BP from  $142 \pm 1$  to  $119 \pm 2$  mm Hg after the fifth daily injection of the bufadienolide. This antihypertensive action of RBG continued so that by the end of the experiment, the mean BP was identical to that in the sham-operated group of animals: SHM group  $116 \pm 2$  mm Hg; NDSR group  $119 \pm 3$  mm Hg (p > 0.05). There were no differences in BP values among any of the groups at t<sub>0</sub> (fig. 1). Furthermore, there were no changes in BP in the SHM group, as the experiment proceeded (fig. 1).



**Fig. 2.** Protein excretion in the five experimental groups of animals. NDS and ANG groups demonstrated an increase in protein excretion (<sup>a</sup> p < 0.05 and p < 0.001, respectively) versus the SHM group. While RBG lowered the protein excretion numerically but not statistically in the NDSR rats (<sup>b</sup> p > 0.05 for NDSR vs. NDS group), it had no effect on this parameter in the ANG group of animals (<sup>c</sup> p > 0.05 for ANGR vs. ANG).

The protein excretion (fig. 2) in the SHM and NDSR groups did not differ from each other: SHM group  $4.5 \pm 0.5$  mg/24 h and NDSR group  $7.8 \pm 0.6$  mg/24 h (p > 0.05). But the value obtained for the NDS group was more than twice that for the SHM group:  $9.1 \pm 1.0$  mg/24 h (p < 0.05). There was a numerical decline in the protein excretion in the NDS rats treated with RBG (NDSR group; from  $9.14 \pm 1.04$  to  $7.80 \pm 0.60$  mg/24 h) which did not reach statistical significance. The mean value for the ANG group of  $19.5 \pm 1.0$  mg/24 h was statistically significantly different from the value of the SHM and NDS groups: p < 0.001 for ANG group versus SHM group and p < 0.01 versus NDS group. However, RBG exerted no influence on the angiotensin-infused animals:  $20.6 \pm 1.0$  mg/24 h (p > 0.05, ANGR group vs. ANG group).

As shown in figure 3, the angiotensin-II-induced rat hypertension is associated with marked oxidative stress. Using the bioluminescent assay with L-012, we verified that angiotensin II administration leads to a dramatic 5.5-fold increase in aortic superoxide levels and found that the BP elevation in the VE model of hypertension correlated with increased superoxide formation ( $0.116 \pm 0.008$  RLU/ $\mu$ g dry tissue for the NDS group vs.  $0.029 \pm 0.007$  RLU/ $\mu$ g dry tissue for the SHM group, p < 0.001). RBG injection to ANG rats had no effect on the superoxide levels ( $0.158 \pm 0.059$  RLU/ $\mu$ g dry tissue for the ANGR group vs.  $0.158 \pm 0.027$  RLU/ $\mu$ g dry tissue for the ANG group, p > 0.05). However, RBG completely eliminated



**Fig. 3.** Aortic superoxides in experimental essential hypertension. Bioluminescent assay of superoxide levels with L-012. Whole rat aortas were dissected, cut into 2- to 3-mm rings, and incubated with lucigenin derivative L-012. The luminescent signal was collected for 1 min and normalized for aortic ring dry weight. The superoxide levels rose statistically significantly in both NDS rats (<sup>a</sup>  $p < 0.001$  vs. SHM animals) and ANG rats (<sup>a</sup>  $p < 0.001$  vs. SHM group). These levels were reduced to values similar to those of the SHM group by RBG administered to the NDSR animals (<sup>b</sup>  $p < 0.05$  for NDSR vs. NDS rats), but RBG had no effect on the superoxide levels in the aortas of the ANG rats (<sup>c</sup>  $p > 0.05$  for ANGR vs. ANG animals).

**Table 1.** Urinary excretion of angiotensinogen ( $U_{AGT}$ )

Groups	$U_{AGT}$ nmol/day	Number of samples	p
SHM	$2.4 \pm 0.5$	6	
NDS	$2.2 \pm 0.3$	5	SHM vs. NDS: $>0.5$
NDSR	$2.0 \pm 0.2$	10	SHM vs. NDSR: $>0.5$
ANG	$7.1 \pm 0.5$	10	SHM vs. ANG: $<0.001$
ANGR	$7.8 \pm 0.4$	10	SHM vs. ANGR: $<0.001$
			NDS vs. ANG: $<0.001$
			NDS vs. ANGR: $<0.001$
			NDSR vs. ANG: $<0.001$
			NDSR vs. ANGR: $<0.001$

'extra' superoxides generated in the VE-mediated hypertension model ( $0.022 \pm 0.003$  RLU/ $\mu$ g dry tissue for the NDSR group vs.  $0.029 \pm 0.007$  RLU/ $\mu$ g dry tissue for the SHM group,  $p < 0.005$ ).

There were no statistically significant differences between any of the groups of animals for creatinine clearance ( $p > 0.05$  for all group comparisons). The mean values were: SHM group  $2.0 \pm 0.4$  ml/min, NDS group  $1.5 \pm 0.3$

ml/min, NDSR group  $1.4 \pm 0.1$  ml/min, ANG group  $1.4 \pm 0.1$  ml/min, and ANGR group  $1.5 \pm 0.1$  ml/min.

The hematocrit values for the SHM, NDS, and NDSR groups did not differ from each other: SHM group  $0.51 \pm 0.01\%$ , NDS group  $0.50 \pm 0.01\%$ ; NDSR group  $0.49 \pm 0.01\%$ . However, the values for the ANG and ANGR animals of  $0.56 \pm 0.01$  and  $0.54 \pm 0.01\%$ , which did not differ from each other, were statistically significantly higher than those of the former three groups ( $p < 0.001$  in each case). Angiotensin has been shown to induce erythropoiesis, most likely by activation of the angiotensin II type 1a receptor in kidney cells [14].

The urinary excretion of angiotensinogen in SHM, NDS, NDSR, ANG, and ANGR rats was  $2.4 \pm 0.5$ ,  $2.2 \pm 0.3$ ,  $2.0 \pm 0.2$ ,  $7.1 \pm 0.5$ , and  $7.8 \pm 0.4$  nmol/day, respectively. There was no significant change in urinary angiotensinogen excretion observed between SHM and either the NDS or NDSR groups. The levels in ANG and ANGR rats were significantly ( $p < 0.5$ ) increased in comparison with SHM, NDS, and NDSR groups (table 1).

## Discussion

The concept that a circulating humoral substance may be involved in certain forms of hypertension was first raised by Dahl et al. [15], based upon data obtained in parabiosis studies. De Wardener et al. [16] enlarged upon these observations, adding data from cross-circulation experiments. Haddy and Overbeck [17] proposed that a circulating humoral substance, subsequently named 'natriuretic hormone', acted by virtue of its ability to inhibit the  $Na^+/K^+$ -ATPase. Others [18–21] proposed that this inhibition had secondary effects on the  $Na^+/Ca^+$  exchange in vascular smooth muscles, leading to vasoconstriction. These workers isolated an endogenous ouabain-like compound, which they suspected could be the putative natriuretic hormone associated with essential hypertension.

Ouabain, digoxin, and their congeners are cardenolides which are natriuretic, vasoconstrictive, and act as cardiac inotropes. However, another family of cardiac glycosides with properties similar to those of the cardenolides has been identified. These agents, the bufadienolides, are similar structurally to the cardenolides but were isolated originally from venom and skin of the toad *Bufo marinus* [22]. They also circulate endogenously in humans. One of these agents, MBG, has received considerable attention. It has been reported that its levels in plasma are elevated in patients with VE-mediated hypertension [23]. Furthermore, experiments in animals have demonstrated that salt load-

ing results in elevated levels of MBG in the blood [24–26] and in the development of hypertension [27, 28].

RBG is a bufadienolide with striking similarity to MBG [8]. It differs only in that it lacks a hydroxyl group at the 5-beta position. We reasoned that this mild structural dissimilarity to MBG might result in a competition between these two compounds for MBG receptors, the identity of which is currently unknown. We anticipated that the antagonism of the hypertensive action of MBG might be related to interference by RBG with the ability of MBG to inhibit Na<sup>+</sup>/K<sup>+</sup>-ATPase. However, this was not the case [10]. How RBG acts to reduce BP is currently under study in our laboratory.

There is evidence that oxidative stress is involved in the pathophysiology of several forms of hypertension [29–31]. This proved to be the case with regard to the hypertension due to both DOCA-salt and angiotensin (fig. 3). However, RBG had a differential effect on the development of ROS in the two hypertensive groups. We observed that RBG not only reduced BP and proteinuria in the NDS rats, but that it also resulted in a dramatic reduction in the generation of superoxide (fig. 3). It had no such effect in the ANG rats. These data buttress our proposal that there is an important role for MBG in the abnormalities noted in DOCA-salt hypertension, a model of VE-mediated hypertension [32]. Furthermore, this appears not to be the case in at least the angiotensin infusion model of vasoconstrictive hypertension, and perhaps in other forms of this disorder characterized by increased vascular resistance.

The present study demonstrates that urinary excretion of angiotensinogen does not rise in VE-mediated hypertension. The amounts of urinary angiotensinogen measured in this study in the SHM and DOCA-saline animal groups were low (2–7 nmol/day) as compared with the circulating concentrations of angiotensinogen (800–1,000 pmol/ml), suggesting the possibility that urinary angiotensinogen is derived from circulatory angiotensinogen [11]. Angiotensinogen is the only known substrate for renin, and the levels of angiotensinogen in humans and rats are close to the K<sub>m</sub> value for renin [33]. Therefore, changes in either angiotensinogen (substrate) or renin (enzyme) could influence the activity of the renin-angiotensin system, and its upregulation may lead to elevated angiotensin levels [12].

The urinary angiotensinogen excretion significantly increased in the ANG rats in comparison with the SHM and NDS rats. These results are in agreement with the findings of other investigators [11, 12, 33], demonstrating that chronic angiotensin II infusion significantly enhances the renal expression of the angiotensinogen

mRNA levels [34]. Angiotensin II infusion enhances the kidney angiotensin II content irrespective of the plasma angiotensin II concentration [11, 12], because intrarenal angiotensinogen is predominantly localized to proximal tubular cells [34] and because there is evidence of extensive angiotensin II type I receptor expression on proximal tubular cells [35]. It is assumed that, in ANG rats, increases in angiotensin II, either in the glomerular filtrate or the interstitial fluid, activate the angiotensin II type I receptor complex. This enhances the angiotensinogen expression in proximal tubular cells, leading to an increased angiotensinogen secretion. Presumably, increased angiotensinogen secretion from proximal tubular cells into the lumen is responsible for the increased urinary excretion rate of angiotensinogen [11].

In summary, we have demonstrated that RBG, an antagonist of MBG, lowers the BP and reduces proteinuria in a model of VE-mediated hypertension. However, it was ineffective in doing so in animals with a vasoconstrictive form of this disorder. Additionally, RBG dramatically reduced superoxide generation in DOCA-salt hypertension, but not in that due to angiotensin infusion. Moreover, we have determined that VE-mediated hypertension does not result in enhancement of the local renin-angiotensin system. These observations provide additional evidence in favor of the view that MBG plays an important role in the pathogenesis of VE-mediated hypertension. They also support the notion that MBG is not involved in vasoconstrictive forms of elevated BP.

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