

Lifetime Overproduction of Circulating Angiotensin-(1-7) Attenuates Deoxycorticosterone Acetate-Salt Hypertension-Induced Cardiac Dysfunction and Remodeling

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Abstract—We evaluated the development of arterial hypertension, cardiac function, and collagen deposition, as well as the level of components of the renin-angiotensin system in the heart of transgenic rats that overexpress an angiotensin (Ang)-(1-7)-producing fusion protein, TGR(A1-7)3292 (TG), which induces a lifetime increase in circulating levels of this peptide. After 30 days of the induction of the deoxycorticosterone acetate (DOCA)-salt hypertension model, DOCA-TG rats were hypertensive but presented a lower systolic arterial pressure in comparison with DOCA-Sprague-Dawley (SD) rats. In contrast to DOCA-SD rats that presented left ventricle (LV) hypertrophy and diastolic dysfunction, DOCA-TG rats did not develop cardiac hypertrophy or changes in ventricular function. In addition, DOCA-TG rats showed attenuation in mRNA expression for collagen type I and III compared with the increased levels of DOCA-SD rats. Ang II plasma and LV levels were reduced in SD and TG hypertensive rats in comparison with normotensive animals. DOCA-TG rats presented a reduction in plasma Ang-(1-7) levels; however, there was a great increase in Ang-(1-7) (≈ 3 -fold) accompanied by a decrease in mRNA expression of both angiotensin-converting enzyme and angiotensin-converting enzyme 2 in the LV. The mRNA expression of Mas and Ang II type 1 receptors in the LV was not significantly changed in DOCA-SD or DOCA-TG rats. This study showed that TG rats with increased circulating levels of Ang-(1-7) are protected against cardiac dysfunction and fibrosis and also present an attenuated increase in blood pressure after DOCA-salt hypertension. In addition, DOCA-TG rats showed an important local increase in Ang-(1-7) levels in the LV, which might have contributed to the attenuation of cardiac dysfunction and pre-fibrotic lesions. (*Hypertension*. 2010;55:889-896.)

Key Words: angiotensin-(1-7) ■ cardiac remodeling ■ cardiac function ■ hypertension

It is currently accepted that angiotensin (Ang)-(1-7) is involved in the control of cardiovascular function and may present important cardioprotective actions.¹⁻³ Ang-(1-7) is present in the heart, and there is evidence that its production in this tissue depends primarily on angiotensin-converting enzyme (ACE) 2 action on Ang II.^{4,5} Accordingly, overexpression of ACE2 protects the heart against Ang II injuries caused by increase in afterload,^{6,7} whereas inactivation of ACE2 is associated with severely decreased left ventricle (LV) contractile function.⁸ The loss of ACE2 expression leads to Ang II accumulation in tissues,⁹ and it is unclear whether the cardiac dysfunction observed is a result of Ang-(1-7) decreased production or an increase in Ang II.⁸

It has been shown that Ang-(1-7) displays an antiarrhythmic effect,¹⁰ protects the heart from ischemic-reperfusion injuries,¹¹ and preserves the LV function after

myocardial infarction¹² or isoproterenol induced-hypertrophy.¹³ In addition, Grobe et al^{14,15} have shown that 28 days of intravenous infusion of Ang-(1-7) attenuates cardiac fibrosis induced by deoxycorticosterone acetate (DOCA)-salt or Ang II hypertension without altering the degree of cardiac hypertrophy or baseline high blood pressure. More recently, Mercure et al¹⁶ showed that transgenic mice with overexpression of Ang-(1-7) selectively in the heart displayed significantly less ventricle hypertrophy and fibrosis in response to Ang II infusion.

Most of the Ang-(1-7) actions in the heart are mediated by its selective receptor, the G-protein coupled receptor Mas.^{17,18} Mas-deficient mice showed a marked increase in extracellular matrix protein expression and a significant cardiac dysfunction.^{19,20} In addition, Mas receptor mediates the antitrophic and antihypertrophic effects of Ang-(1-7) in cardiac myocytes and fibroblasts, respectively.^{21,22}

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In the present study, we evaluated whether a lifetime increase in circulating levels of Ang-(1-7) that is observed in the transgenic rat that overexpresses an Ang-(1-7)-producing fusion protein, TGR(A1-7)3292 (TG),^{23,24} would alter the development of arterial hypertension, cardiac function, and collagen deposition or the level of components of the renin-angiotensin system after DOCA-salt hypertension.

Methods

An expanded Methods section detailing the techniques and procedures performed is provided as an online Data Supplement. Please see <http://hyper.ahajournals.org>.

Animals

Male Sprague-Dawley (SD) and TG rats 3 to 4 months old were obtained from the transgenic animal facilities of the Federal University of Minas Gerais Hypertension Laboratory. The generation and characterization of this transgenic rat model was described previously by Santos et al.²³ The rats were maintained in a temperature-regulated room (22°C to 24°C) on 12-hour:12-hour light:dark cycles. All of the experimental protocols were approved by our institutional committee that regulates the use of laboratory animals (Comitê de Ética em Experimentação Animal/Federal University of Minas Gerais; protocol No. 67-2007).

DOCA-Salt Hypertension

Rats were nephrectomized (left kidney) under tribromethanol (0.25 g/kg, IP) anesthesia. Part of the animals (DOCA) were implanted with a subcutaneous pellet (Silicone rubber encapsulant, Down-Corning) containing deoxycorticosterone acetate (200 mg/kg; Sigma) and had a solution of 0.9% NaCl and 0.2% KCl to drink for 30 days. Control rats (CTL) were only uninephrectomized. Systolic arterial pressure (SAP) was evaluated by tail-cuff plethysmography (RTBP2000, Kent Scientific) 1 day before and each 10 days of treatment.

Echocardiography

Transthoracic echocardiography was performed in SD and TG rats by the same observer with the use of a SEQUOIA 512 (ACUSON Corp) transducer with a 10- to 13-MHz multifrequencial linear response.

Tissue and Blood Collection

Thirty days after the induction of hypertension, the rats were killed by decapitation and the heart was immediately removed; the atria and right ventricle were dissected free from the LV and discarded. The LV was weighed, sectioned in 3 transversal segments, and quickly frozen on dry ice. Simultaneously, the trunk blood was rapidly collected in polypropylene tubes containing enzymatic inhibitors, as described previously in Santos et al.,²³ and centrifuged. Plasma and heart segments were kept in -80° until assayed.

LV Relative Weight

The ratio of LV weight:tibia length (milligrams per centimeter) was calculated for the assessment of the LV hypertrophy.²⁵

Reverse Transcription and Real-Time PCR

Total mRNA isolation was performed following the TRIzol reagent (Invitrogen Life Technologies), treated with DNase, and reverse transcribed with Moloney murine leukemia virus (Invitrogen). The endogenous S26 ribosomal (internal control) and genes presented in LV, as collagen type I and III, ACE, ACE2, Mas, and Ang II type 1 (AT₁) receptor cDNA were amplified using specific primers and SYBR Green reagent (Applied Biosystems) in an ABI Prism 7000 platform (Applied Biosystems), as described previously.²⁶

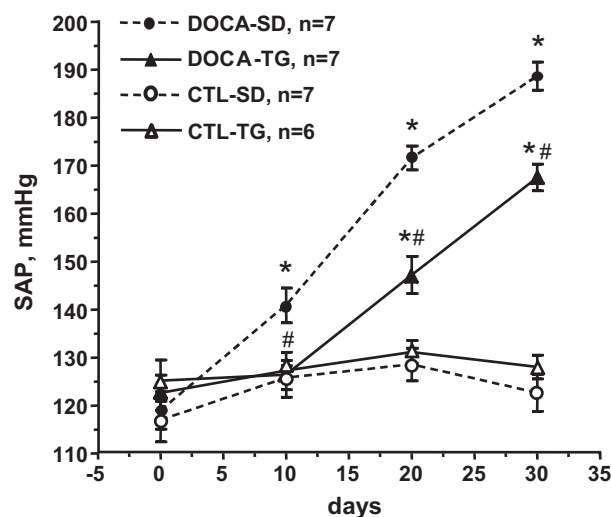


Figure 1. SAP (in millimeters of mercury) before (time 0) and each 10 days after induction of hypertension (DOCA) or CTL in SD and TG rats. Values are mean \pm SE. * P <0.05 in comparison with respective before and # P <0.05 in comparison with DOCA-SD (2-way ANOVA followed by Bonferroni test).

Immunohistochemical Analysis

Immunofluorescence labeling and qualitative confocal microscopy were used to investigate the distribution of collagen types I and III in LV of SD and TG rats after DOCA-salt hypertension or CTL.

Ang-(1-7) and Ang II Measurements

The LV was homogenized in 4 mol/L of guanidine thiocyanate/1% trifluoroacetic acid (vol/vol; 5 mL for each tissue) in water and then processed as described previously.²⁷ Plasma and LV peptides were extracted onto Bond-Elut phenylsilane cartridges (Varian) followed by Ang-(1-7) and Ang II levels measurement by radioimmunoassay, as described previously by Santos et al.²³

Statistical Analysis

Data are expressed as mean \pm SE. Differences among the groups were assessed by 2-way ANOVA followed by the Bonferroni test. The statistical analysis was performed with GraphPad Prism software (version 4.0), and the level of significance was set at P <0.05.

Results

Arterial Pressure Levels

TG and SD rats presented similar SAP before treatment. After 30 days, DOCA-TG rats developed a lower SAP (168 ± 3 mm Hg; $n=7$) in comparison with DOCA-SD (188 ± 3 mm Hg; $n=7$). In addition, DOCA-TG presented a significantly increased SAP only on day 20 (148 ± 4.5 versus 129 ± 2 mm Hg, in CTL-TG rats; P <0.05), whereas DOCA-SD rats were hypertensive on day 10 after the surgery (141 ± 4 versus 126 ± 2 mm Hg, CTL-SD rats; P <0.01). No difference in SAP was observed between CTL-SD and CTL-TG rats during the 30-day period (Figure 1).

LV Hypertrophy

The ratio of LV weight:tibia length of DOCA-SD rats (0.2499 ± 0.0060 mg/cm; $n=7$; Table) was significantly higher in comparison with CTL rats (0.2170 ± 0.0060 mg/cm; $n=5$; Table). DOCA-TG rats presented a lower hypertrophic response (0.227 ± 0.006 mg/cm, $n=6$, versus 0.2040 ± 0.008 mg/cm, $n=6$, in CTL-TG rats; Table). In keeping with these

Table. Parameters of LV Hypertrophy Obtained by LV Weight and Transthoracic Echocardiography in SD and TG Rats 30 Days After Hypertension (DOCA) or CTL

Parameters	CTL-SD	DOCA-SD	CTL-TG	DOCA-TG
LVW/TL, mg/cm	0.217±0.006	0.2499±0.006*	0.204±0.008	0.227±0.006
RWT	0.398±0.018	0.464±0.018*	0.428±0.023	0.476±0.021
IVS (%) THCK	54.0±3.9	73.6±3.4*	60.7±2.9	61.8±4.4
LVPW (%) THCK	57.5±4.1	62.4±3.4	51.6±1.4	61.6±3.9*

LVW/TL indicates LV weight/tibia length; RWT, relative wall thickness; IVS (%) THCK, percentage of the systolic and diastolic difference of interventricular septum thickness; LVPW (%) THCK, percentage of the systolic and diastolic difference of posterior wall thickness. Values are mean±SE.

* $P<0.05$ vs respective CTL (2-way ANOVA followed by Bonferroni test).

data, DOCA-SD rats presented a significant increased in relative wall thickness ($0.46±0.02$ versus $0.40±0.01$ in CTL-SD; $P<0.05$; Table) and interventricular septum hypertrophy ($73.6±3.4%$ versus $54.0±3.9%$ in CTL-SD rats; $P<0.05$; Table), whereas no significant differences were observed in DOCA-TG rats. DOCA treatment in TG rats, however, induced an increase in posterior wall thickness of LV ($62±3.9%$ versus $52±1.4%$ in CTL-TG rats; Table).

LV Systolic and Diastolic Function

Thirty days after induction of the hypertension, SD animals showed a significant increase in the ejection fraction ($79±1%$, $n=12$, versus $74±1%$, $n=9$, in CTL-SD rats; $P<0.05$; Figure 2) and an increase in the fractional shortening

($43±1%$, $n=10$, versus $37±1%$, $n=8$; $P<0.05$; Figure 2) that is consistent with the LV hypertrophy and indicates that the DOCA-SD rats presented a compensated systolic function. In contrast, DOCA-TG rats did not show alterations in these parameters. In addition, DOCA-SD rats presented signs of diastolic dysfunction observed by a lower aorta:left atrium ratio ($0.90±0.03$ versus $0.99±0.01$ in CTL-SD rats; $P<0.05$; Figure 2) accompanied by an increase in isovolumetric relaxation time ($30.9±1.26$ versus $26.3±0.67$ ms; $P<0.05$; Figure 2). No significant changes in diastolic function were observed in DOCA-TG rats.

Cardiac Collagen Deposition

As shown in Figure 3, the preserved cardiac function in TG rats was accompanied by attenuation in mRNA expression for collagen type I and collagen type III ($1.65±0.27$ and $1.38±0.17$ arbitrary units [AU], respectively) in comparison with the increased levels observed in DOCA-SD rats ($2.31±0.35$ and $1.91±0.22$ AU, respectively). No significant differences were observed in mRNA expression of collagen type I and III in the LV of CTL-SD and CTL-TG rats (Figure 3).

Plasma Angiotensin Levels

As expected, TG rats presented a significantly higher Ang-(1-7)-immunoreactivity in plasma ($27.71±4.16$ pg/mL; $n=11$; Figure 4) as compared with SD rats ($16.88±2.28$ pg/mL; $n=9$; $P<0.05$; Figure 4). Surprisingly, the increased Ang-(1-7) plasma levels of TG rats were significantly reduced after DOCA treatment ($14.30±1.48$ versus $27.71±4.16$ pg/mL in CTL-TG rats; $P<0.01$), reaching similar levels of CTL-SD and DOCA-SD rats (Figure 4). No

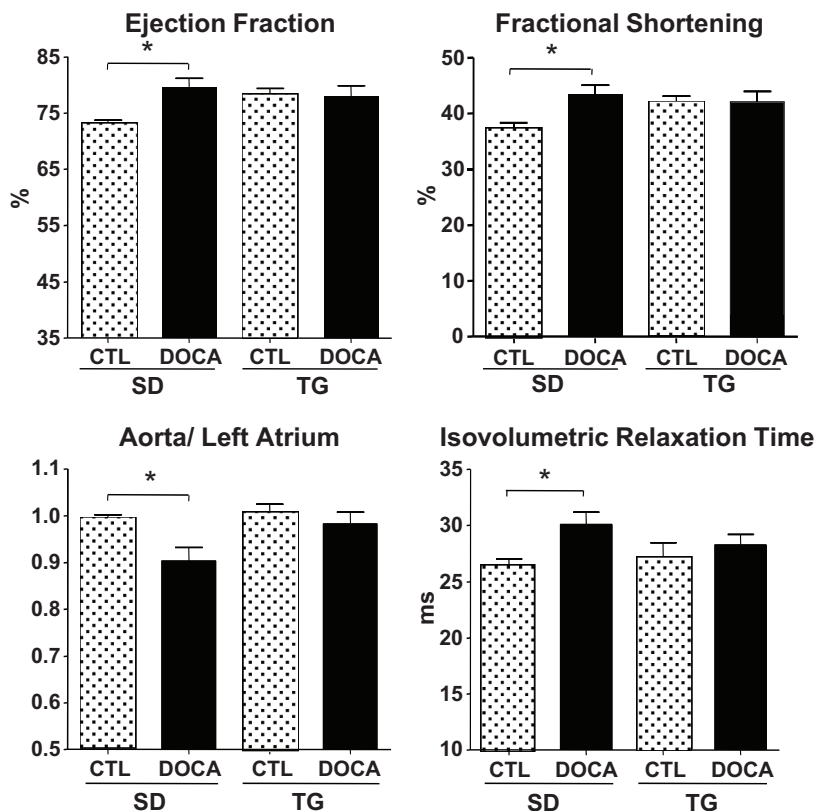


Figure 2. Echocardiography parameters obtained in SD and TG rats after DOCA-salt treatment or CTL. Values are mean±SEM. * $P<0.05$ (2-way ANOVA followed by Bonferroni test).

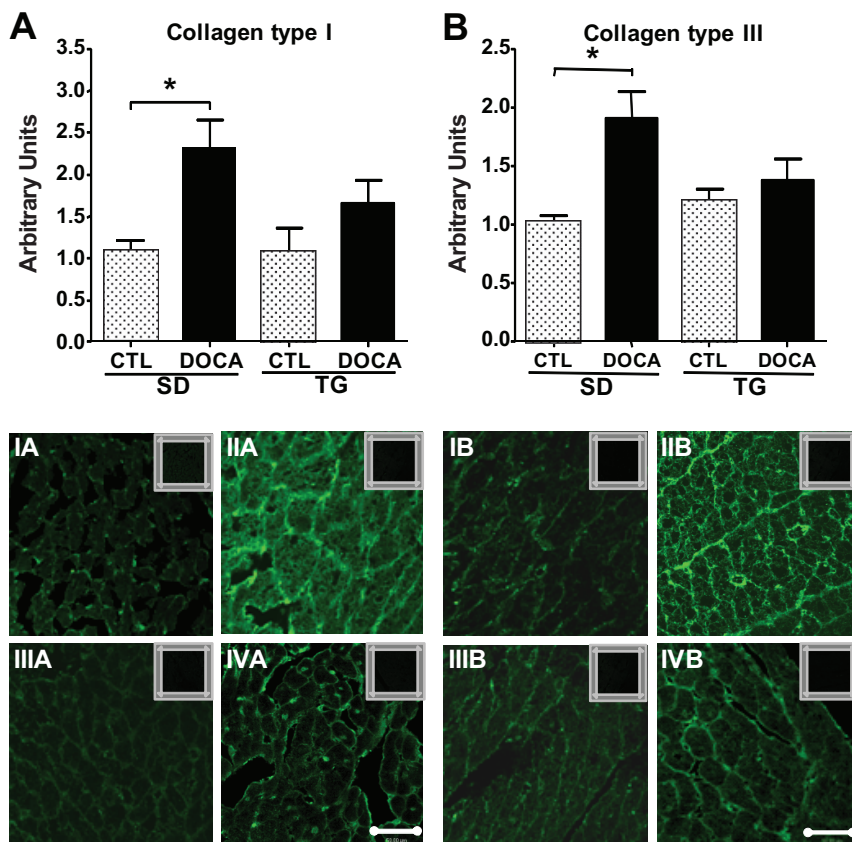


Figure 3. LV mRNA expression (arbitrary units) of collagen type I (A) and type III (B) obtained by real-time PCR in SD and TG rats after 30 days of DOCA-salt or CTL. Below the graphs, representative images of LV sections showing immunofluorescent localization obtained by confocal microscopy. Images IA, IIA, IIIA, and IVA are collagen type I staining in CTL-SD, DOCA-SD, CTL-TG, and DOCA-TG rats, respectively. Images IB, IIB, IIIB, and IVB are collagen type III staining in CTL-SD, DOCA-SD, CTL-TG, and DOCA-TG rats, respectively. Gray box at the top right of image shows the level of immunostaining obtained when the primary antibody is omitted from the incubation procedure. The line in the bottom right represents 50 μm . Values are mean \pm SEM. * $P < 0.05$ (2-way ANOVA followed by Bonferroni test).

significant difference was observed in the plasma Ang II levels between CTL-SD and CTL-TG rats. As expected, DOCA treatment induced a significant decrease in the plasma Ang II level in both SD (10.07 ± 0.79 versus 17.82 ± 2.63 pg/mL in CTL-SD rats; $P < 0.05$) and TG rats (12.24 ± 0.92 versus 18.30 ± 2.45 pg/mL in CTL-TG rats; $P < 0.05$; Figure 4).

LV Angiotensin Levels

As shown in Figure 4, Ang-(1-7)-immunoreactivity was similar in the LV of CTL-TG and CTL-SD rats. There was a tendency for lower levels of Ang II ($\approx 40\%$) in the LV of CTL-TG rats in comparison with CTL-SD rats (2.71 ± 0.59 pg/mg of protein versus 4.13 ± 0.27 pg/mg of protein, respectively; $P > 0.05$; Figure 4). After DOCA treatment, SD and TG rats presented reduced cardiac Ang II (1.78 ± 0.34 pg/mg of protein and 1.23 ± 0.23 pg/mg of protein, respectively) in comparison with CTL-SD and CTL-TG rats (4.13 ± 0.27 pg/mg of protein and 2.71 ± 0.59 pg/mg of protein, respectively; $P < 0.05$; Figure 4). Strikingly, however, after DOCA treatment there was a significant ≈ 3 -fold increase in Ang-(1-7) only in the LV of TG rats (4.69 ± 0.78 versus 1.53 ± 0.09 pg/mg of protein in CTL-TG rats; $P < 0.01$; Figure 4).

mRNA Expression of Renin-Angiotensin System Components in the LV

To better understand the increase in cardiac levels of Ang-(1-7) in TG rats, we evaluated the mRNA expression of the main angiotensin-forming enzymes, ACE and ACE2, in the LV. Interestingly, CTL-TG rats presented higher mRNA

expression of cardiac ACE2 (2.49 ± 0.28 versus 1.00 ± 0.23 AU in CTL-SD rats; $P < 0.001$). In addition, after 30 days of DOCA treatment, there was a significant reduction in ACE2 mRNA expression in the LV of TG rats (1.18 ± 0.23 versus 2.49 ± 0.28 AU in CTL-TG rats; $P < 0.01$), to a level comparable to that observed in DOCA-SD rats (1.15 ± 0.15 AU; Figure 5). In addition, DOCA-TG rats showed a $\approx 60\%$ lower ACE mRNA expression in the LV (0.35 ± 0.08 versus 0.92 ± 0.22 AU in CTL-TG rats; $P < 0.05$; Figure 5). SD rats did not show alteration in ACE mRNA expression.

The expression of the Mas and AT_1 receptors in the LV was also evaluated. No significant difference was observed in the mRNA expression of Mas or AT_1 receptors in the LV of control or hypertensive SD and TG rats (Figure 5). However, DOCA-TG presented an $\approx 25\%$ decrease (not significant) in AT_1 receptor expression in comparison with CTL rats (0.78 ± 0.08 versus 1.05 ± 0.14 AU, respectively; $P > 0.05$; Figure 5).

Discussion

The data presented in this study showed that transgenic rats with overexpression of an Ang-(1-7)-producing fusion protein that induces a lifetime increase in circulating levels of Ang-(1-7) are protected not only against cardiac dysfunction and fibrosis but also presented an attenuated increase in blood pressure after DOCA-salt hypertension. More interestingly, these effects were associated with a local increase of ≈ 3 -fold in Ang-(1-7)-immunoreactivity in the LV.

TG rats had attenuation in the development of arterial hypertension, cardiac collagen deposition, and LV dysfunc-

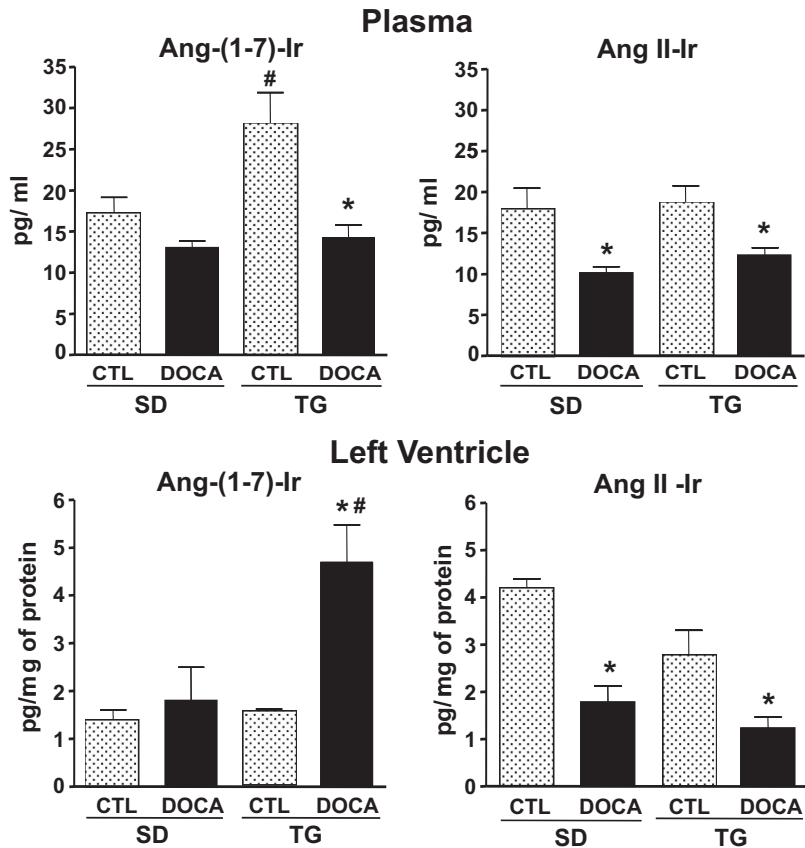


Figure 4. Ang-(1-7) and Ang II immunoreactivity levels determined by radioimmunoassay in the plasma and in the LV of SD and TG rats after 30 days of DOCA-treatment or CTL. Values are mean \pm SEM. ^{*} $P < 0.05$ in comparison with respective CTL and [#] $P < 0.05$ in comparison with SD-CTL (2-way ANOVA followed by Bonferroni test).

tion after induction of the DOCA-salt hypertension. Previous studies by Grobe et al^{14,15} have shown that Ang-(1-7) attenuates cardiac fibrosis induced by DOCA-salt or Ang II hypertension. In addition, transgenic mice with overexpression of Ang-(1-7) selectively in the heart displayed significant less ventricle hypertrophy and fibrosis in response to Ang II infusion.¹⁶ Our data extended these findings by showing that lifetime increase in circulating Ang-(1-7), using a transgenic rat model, was also capable of changing local levels of Ang-(1-7) in the heart, which may have contributed to the antifibrotic and antihypertrophic effects observed after the induction of hypertension. Our results suggest additionally that long-term increase in circulating Ang-(1-7) may not induce tachyphylaxis.

Other studies have shown that Ang-(1-7) concentration in the heart may increase in different situations, such as after the myocardium infarction,⁴ in heart failure,⁵ or after physical training.²⁸ We showed that, parallel to the increase in Ang-(1-7) in the heart of TG rats, there was a decrease in Ang II levels, resulting in an ≈ 6 -times increase in the ratio Ang-(1-7):Ang II in the LV (3.8 versus 0.6 in CTL-TG rats), which may have helped to decrease the deleterious effect of DOCA-salt hypertension in the heart of TG rats. The decrease in Ang II levels in the LV after DOCA-salt is in agreement with the observation of a decrease in myocardial renin and angiotensinogen in this model of hypertension.²⁹

It is interesting that normotensive CTL-TG in comparison with CTL-SD rats presented an increased ACE2 mRNA expression accompanied by similar levels of Ang-(1-7) and a

tendency for lower Ang II levels in the LV. These data are consistent with those obtained in previous studies. SD rats submitted to chronic subcutaneous infusion of Ang-(1-7) presented a decrease in Ang II accompanied by an increase in ACE2 mRNA expression in the LV.³⁰ Ang II possesses an inhibitory effect on ACE2 mRNA expression in astrocytes and cardiomyocytes cultures,³¹ suggesting that a tissue decrease in Ang II could contribute to increased ACE2. In addition, TG rats presented a reduction in angiotensinogen mRNA expression in the LV.³² Thus, it is possible that the lower Ang II was because of the combination of lower angiotensinogen and the increased ACE2. On the other hand, the increased ACE2 could compensate for the lower local angiotensinogen and could, therefore, maintain Ang-(1-7) levels in the LV of these rats.

On the basis of the increased ACE2 expression in the CTL-TG rats, we would expect that the increased level of Ang-(1-7) in the LV of TG rats after DOCA-salt hypertension could also be because of an increased level of ACE2. However, in DOCA-TG rats, ACE2 mRNA expression was decreased to a level comparable to that found in CTL-SD or DOCA-SD rats. This is an intriguing finding, because ACE2 is considered to be the main enzyme involved with the Ang-(1-7) formation from Ang II.^{5,8} On the other hand, DOCA-TG rats also presented an $\approx 60\%$ lower ACE mRNA expression in the LV. The lower ACE may be importantly related to the increased level of Ang-(1-7), because ACE is the major enzyme also responsible for the hydrolysis of this peptide.³³

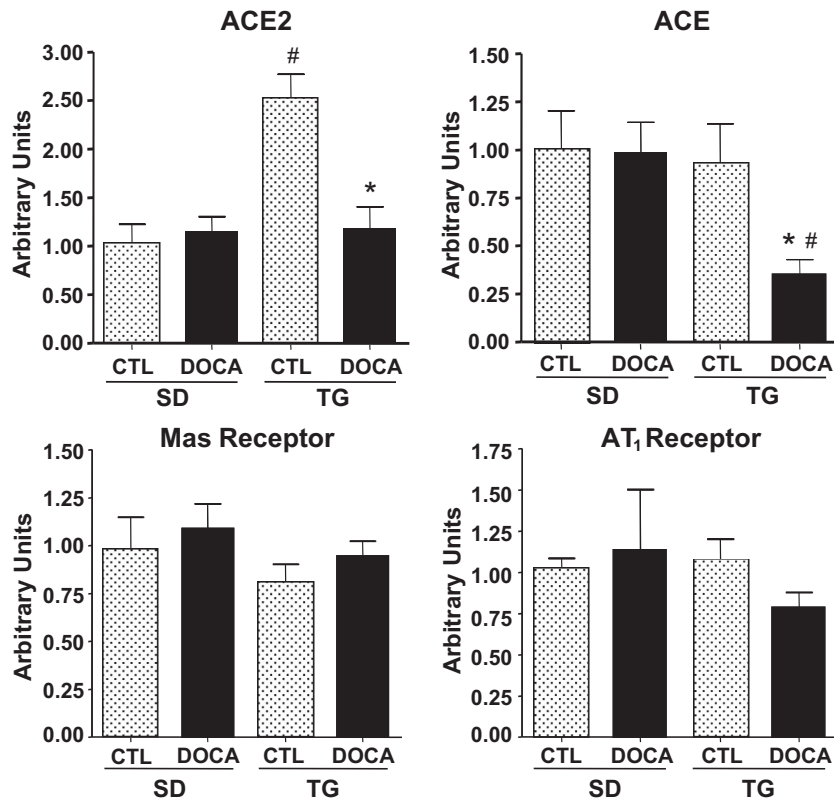


Figure 5. LV mRNA expression (arbitrary units) by real-time PCR of ACE2 and ACE and the angiotensin receptors, Mas and AT₁, in SD and TG rats 30 days after DOCA-treatment or CTL. Values are mean±SE. * $P < 0.05$ in comparison with respective CTL and # $P < 0.05$ in comparison with SD-CTL (2-way ANOVA followed by Bonferroni test).

Other possibilities are the stimulation of alternative pathways for Ang-(1-7) formation in the heart after DOCA treatment. Campbell et al,²⁷ measuring Ang peptides (Ang I, Ang II, Ang-[1-9], and Ang-[1-7]) in arterial and coronary sinus blood of patients with heart failure receiving ACE therapy, suggested that endopeptidases may be involved in Ang-(1-7) formation from Ang I independent of ACE2. In addition, it was shown recently that Ang-(1-12) can be an important substrate for angiotensin peptide formation in the rat heart.³⁴ Therefore, the increase in Ang-(1-7) in the heart of DOCA-TG rats could be because of the decreased ACE or a change in other enzymes involved in Ang-(1-7) generation, such as neutral endopeptidase, prolyl-endopeptidase, and prolyl-carboxypeptidase. Additional studies will be necessary to evaluate whether the accumulation of Ang-(1-7) in the LV of DOCA-TG is solely attributable to the decrease in ACE in association with normal ACE2 expression or may also be because of the activation of alternative enzymatic pathways.

On the basis of the angiotensin levels, we next assessed the mRNA expression of the angiotensin receptors, AT₁ and Mas, in the LV. In our study, no change in the expression of Mas receptor mRNA was observed in TG rats associated with the increase in Ang-(1-7) in the LV. This result is in contrast to a recent study in our laboratory that showed a simultaneous increase in the expression of Mas and Ang-(1-7) levels in the LV of spontaneously hypertensive rats submitted to exercise training.²⁸ Similar to the expression of Mas, the expression of AT₁ receptors in the LV was not different in SD or TG rats after DOCA hypertension. Other studies in the literature showed that infusion of Ang-(1-7) reduces the levels of AT₁ receptors in vascular smooth muscle cells³⁵ and kidney.³⁶

Thus, it is possible that the prolonged increase in Ang-(1-7) has limited the increase in AT₁ receptor expression that is generally observed after DOCA-salt hypertension.^{37,38}

Ang-(1-7) was extensively shown to possess cardioprotective effects, preventing cardiac remodeling in vitro^{21,22} and in vivo.^{12-16,20,23} In accordance, our data showed that DOCA-TG rats presented decreased deposition (by immunofluorescence and mRNA expression) of collagen type I and type III in the LV, in comparison with DOCA-SD rats, suggesting that Ang-(1-7) can modulate the cardiac deposition of proteins of the interstitial matrix. The effects of Ang-(1-7) on cardiac collagen deposition are likely to have contributed to the preservation of the cardiac function of DOCA-TG rats against the hypertensive state. Our results showed that, 30 days after the induction of DOCA-salt hypertension, although systolic cardiac function was compensated, DOCA-SD presented LV hypertrophy accompanied by a reduction in the aorta/left atrium ratio, indicating an increased left atrium residual volume probably caused by impaired ventricle relaxation. In addition, DOCA-SD rats presented an increase in isovolumetric relaxation time, suggesting a slower initial relaxation. In contrast, DOCA-TG rats did not show LV hypertrophy or signs of diastolic dysfunction. Interestingly, DOCA-TG rats presented a significant increase in posterior wall thickness, which may have contributed to preserve the systolic function of these animals against the increased afterload induced by hypertension, without important interference in the diameter of the ventricular chamber.

Recently, a study of our group investigated the signaling pathways involved in Ang-(1-7) cardioprotection, showing that the antihypertrophic effect of this peptide is related to its

modulation on prohypertrophic gene transcription.³⁹ Ventricular myocytes from TG rats were protected from Ang II–induced pathological remodeling characterized by fetal gene expression, Ca²⁺ signaling dysfunction, GsK3 β inactivation, and nuclear factor of activated T-cells nuclear accumulation. In addition, cardiomyocytes from these TG animals infused with Ang II presented an increase in the expression of NO synthase, and the antihypertrophic effect was prevented by inhibitors of NO synthase, N^G-nitro-L-arginine methyl ester, or ODQ (1*H*-1,2,4-oxadiazolo[4,2-*a*]quinoxalin-1-one), suggesting a role for NO/cGMP as mediators of the beneficial effects of Ang-(1-7) in cardiac cells.

In our study, DOCA-TG rats developed a lower SAP in all of the periods evaluated after the induction of the hypertension. This result is in contrast to the studies of Grobe et al^{14,15} that observed that Ang-(1-7) infusion during 4 weeks had no effect on the increased SAP induced by DOCA-salt or Ang II infusion. These contrasting results may be related to distinct effects induced by the relatively short time infusion in comparison with the lifetime increase in Ang-(1-7) that is observed in TG rats. On the other hand, Mercure et al¹⁶ showed that transgenic mice that overexpress Ang-(1-7) selectively in the heart present similar hypertension induced by Ang II infusion that controls animals. However, in this study, circulating levels of Ang-(1-7) were not altered. Our data are in keeping with the well-known vasodilatory action of Ang-(1-7)^{40,41} and the data of a previous study showing that these transgenic rats presented a decrease in the total peripheral resistance because of a vasodilatory effect of Ang-(1-7) in different vascular beds.⁴¹ Other studies have shown that Ang-(1-7) can decrease blood pressure in spontaneously hypertensive rats.⁴² Thus, it is possible that the lifetime increase in Ang-(1-7) has contributed to attenuate the development of high blood pressure after DOCA-salt.

One may argue that the cardiac beneficial effects observed in our TG rats are solely attributed to the attenuated hypertension that these animals developed after DOCA-salt treatment. It is well known that a lower blood pressure results in a reduced afterload, which, in turn, could contribute to the reduced cardiac remodeling observed in DOCA-TG rats. However, taking into account the data of Grobe et al^{14,15} and Mercure et al,¹⁶ the attenuation of the hypertension appears to be only a minor component of the decrease in cardiac remodeling observed in TG rats.

Perspectives

The results of the present study showed that lifetime increase in circulating Ang-(1-7), using a transgenic rat model, attenuated arterial hypertension, cardiac collagen deposition, and dysfunction induced by DOCA-salt hypertension. More importantly, the chronic increase in plasma Ang-(1-7) was capable of changing local levels of Ang-(1-7) in the heart, which may have contributed to its antifibrotic and antihypertrophic effects. These data reinforce that the development of pharmacological strategies that lead to a systemically accumulation of Ang-(1-7) may be an important alternative for the treatment of cardiovascular diseases.

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Disclosures

None.

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Lifetime Overproduction of Circulating Angiotensin-(1-7) Attenuates Deoxycorticosterone Acetate-Salt Hypertension-Induced Cardiac Dysfunction and Remodeling

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**LIFETIME OVERPRODUCTION OF CIRCULATING ANGIOTENSIN-(1-7)
ATTENUATES DOCA-SALT HYPERTENSION-INDUCED CARDIAC
DYSFUNCTION AND REMODELING**

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Running Title: Ang-(1-7) and cardiac function and remodeling

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METHODS (supplement)

Indirect arterial pressure measurement

Systolic arterial pressure (SAP) was evaluated by tail cuff plethysmography (RTBP2000, Kent Scientific) one day before and each 10 days of treatment. At least three separated SAP measures were obtained for each animal in each day of measurement

Echocardiography. Echocardiographic indices, obtained according to the recommendations of the American Society of Echocardiography Transthoracic. Echocardiography was performed in SD and TG rats by the same observer with the use of a SEQUOIA 512 (ACUSON Corp) transducer with 10- to 13-MHz multifrequencial linear response. All measurements were based on the average of 3 consecutive cardiac cycles. Rats were anesthetized with a mixture of ketamine hydrochloride, 50 mg/ Kg and xylazine, 10 mg/ Kg ip. Wall thickness and LV dimensions were obtained from a short axis view at the level of the papillary muscles.

Reverse transcription and real-time PCR. Total RNA isolation was performed following the TRIzol reagent method (Invitrogen Life Technologies) according to the manufacturer's protocol. RNA samples (2µg) were treated with DNase to eliminate genomic DNA present in the samples. The mRNA expression was assessed by RT-PCR after reverse transcription with MML-V (Moloney murine leukemia virus) (Invitrogen). The endogenous S26 ribosomal (internal control) and genes presented in LV as collagen type I and III, ACE, ACE2, Mas and AT₁ receptor cDNA were amplified using specific primers (Table I) and SYBR Green reagent (Applied Biosystems). The reactions were performed using 40 cycles and annealing temperature of 60°C (ABI Prism 7000, Applied Biosystem).

Immunohistochemical Analysis. Immunofluorescence labeling and qualitative confocal microscopy were used to investigate the distribution of collagen types I and III in LV of SD and TG rats after DOCA-salt hypertension or sham-surgery. The LV was cryofixed in a -80°C solution of 80% methanol and 20% dimethyl sulfoxide for 7 days. Samples were rehydrated through a graded series of ethanol to PBS following by washing in 30% sucrose and embedded in Tissue Teck (OCT compound, Miles, USA). Sections of 10 µm thick were mounted on slides obtained in the cryostat in -20°C. Tissue sections were washed in water and PBS for 10 min and then incubated in blocking solution (1% BSA and 0.1% Tween 20 in PBS) at room temperature for 1 h. Sections were incubated overnight at 4 °C with one of the following primary antibodies: rabbit anti-human collagen type I (1:200; Research Diagnostics Inc.), rabbit anti-human collagen type III (1:200; Research Diagnostics Inc.). Information from the suppliers indicates that each of these antibody reacts specifically with both the human and rat antigens. All antibodies were diluted with 1:10 diluted blocking solution. After 4–5 rinses in PBS, donkey anti-rabbit IgG conjugated with Alexa Fluor 488 (Molecular Probes Laboratories, Inc., USA) was added and incubated for 1 h in the dark at room temperature. Following washes with PBS, sections were mounted and viewed with a laser scanning confocal microscope (Zeiss LSM 510Meta). All confocal settings (aperture, gain and laser power) were determined at the beginning of the imaging session and then held constant during the session.

Blood sample collection. At the end of the 30-day period, the rats were killed by decapitation and the trunk blood was rapidly collected into chilled tubes containing 1 mM p-hydroxymercuribenzoate, 30 mM of 1,10-phenanthroline, 1 mM PMSF, 1 mM pepstatin A, and 7.5% EDTA (50µl/ ml of blood). After centrifugation, plasma samples were frozen in dry ice and stored at -20°C.

LV collection. The LV was homogenized in 4 mol/l guanidine thiocyanate (GTC)/1% trifluoroacetic acid (TFA) (vol/vol; 5 ml for each tissue) in water and then processed as described previously by Campbell et al.¹

Peptide extraction and Radioimmunoassay (RIA). Peptides present in samples of plasma and LV were extracted onto Bond-Elut phenylsilane cartridges (Varian, USA). The columns were preactivated by sequential washes with 10 ml of 99.9% acetonitrile/0.1% heptafluorobutyric acid (HFBA) and 10 ml of 0.1% HFBA. Sequential washes with 10 ml of 99.9% acetonitrile/0.1% HFBA, 10 ml of 0.1% HFBA, 3 ml of 0.1% HFBA containing 0.1% BSA, 10 ml of 10% acetonitrile/0.1% HFBA, and 3 ml of 0.1% HFBA were used to activate the columns. After sample application, the columns were washed with 20 ml of 0.1% HFBA and 3 ml of 20% acetonitrile/0.1% HFBA. The adsorbed peptides were eluted with 3 ml of 99.9% acetonitrile/0.1% HFBA into polypropylene tubes rinsed with 0.1% fat-free BSA. After evaporation, angiotensin-(1-7) and angiotensin II levels were measured in plasma and LV extracts by RIA, as previously described in Botelho et al² and Santos et al.³ Protein concentration in the heart homogenates was determined by the Bradford method.⁴

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Table S1 . Primers sequence used to perform real-time PCR

Gene	Sequence (5' to 3') forward	Sequence (5' to 3') reverse
Collagen type I	TGT TCA GTC TTG TGG ACC TC	CCT TAG GC ATT GTG TAT GC
Collagen type III	CTT ATT TTG GCA CAG TCC	TTT GAC ATG GTT CTG GCT TCC
ACE	TGT TGG TGA GCT CTG GGT C	CAG GAA GCT GAA GAC CTG TC
ACE2	CAG GAA GCT GAA GAC CTG TC	GAC TGT TCT TCA TAA AAG GCA
Mas receptor	ACT GTC GGG CGG TCA TCA TC	GGT GGA GAA AAG CAA GGA GA
AT ₁	TCT CAG CAT CGA TCG CTA CCT	AGG CGA GAC TTC ATT GGG TG
S26	CGA TTC CTG ACA ACC TTG CTA TG	CGT GCT TCC CAA GCT CTA TGT