Beneficial Effects of Angiotensin-(1–7) Against Deoxycorticosterone Acetate–Induced Diastolic Dysfunction Occur Independently of Changes in Blood Pressure

Pedro W. Machado de Almeida, Marcos Barrouin Melo, Ricardo de Freitas Lima, Mariana Gavioli, Nivia M. Santiago, Leonardo Greco, Itamar C.G. Jesus, Eduardo Nocchi, Amanda Parreira, Marcia N.M. Alves, Luciana Mitraud, Rodrigo Ribeiro Resende, Maria José Campagnole-Santos, Robson Augusto Souza dos Santos, Silvia Guatimosim

Abstract—Mineralocorticoids have been implicated in the pathogenesis of diastolic heart failure. On the contrary, angiotensin (Ang)–(1–7) has emerged as a potential strategy for treatment of cardiac dysfunction induced by excessive mineralocorticoid receptor activation. A critical question about the cardioprotective effect of Ang–(1–7) in hypertensive models is its dependence on blood pressure (BP) reduction. Here, we addressed this question by investigating the mechanisms involved in Ang–(1–7) cardioprotection against mineralocorticoid receptor activation. Sprague-Dawley (SD) and transgenic (TG) rats that overexpress an Ang–(1–7) producing fusion protein (TG(A1–7)3292) were treated with deoxycorticosterone acetate (DOCA) for 6 weeks. After treatment, SD rats became hypertensive and developed ventricular hypertrophy. These parameters were attenuated in TG-DOCA. SD-DOCA rats developed diastolic dysfunction which was associated at the cellular level with reduced Ca2+ transient. Oppositely, TG-DOCA myocytes presented enhanced Ca2+ transient. Moreover, higher extracellular signal–regulated kinase phosphorylation, type 1 phosphatase, and protein kinase Cε levels were found in SD-DOCA cells. In vivo, pressor effects of DOCA can contribute to the diastolic dysfunction, raising the question of whether protection in TG was a consequence of reduced BP. To address this issue, BP in SD-DOCA was kept at TG-DOCA level by giving hydralazine or by reducing the DOCA amount given to rats (Low-DOCA). Under similar BP, diastolic dysfunction and molecular changes were still evident in DOCA-hydralazine and SD-low-DOCA, but not in TG-DOCA. In conclusion, Ang–(1–7) protective signaling against DOCA-induced diastolic dysfunction occurs independently of BP attenuation and is mediated by the activation of pathways involved in Ca2+ handling, hypertrophy, and survival.

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Key Words: angiotensins • hypertrophy, left ventricular • myocytes, cardiac

Clinical studies have linked aldosterone excess to the development and progression of several different cardiovascular disease processes.1 In the heart, aldosterone binds both mineralocorticoid receptor (MR) and glucocorticoid receptor triggering the activation of different signaling pathways.2 Activation of MR has been implicated in the development of diastolic dysfunction,4 and its conditional expression in the heart leads to arrhythmias.5 Di et al5 have shown that combined increases of systemic angiotensin II (Ang II) and cardiac MR signaling culminated in additive effects in the heart leading to pronounced left ventricular hypertrophy (LVH) and diastolic dysfunction. Conversely, Ang–(1–7), which is an active peptide of renin–angiotensin system, is known to protect the heart against deoxycorticosterone acetate (DOCA)-induced fibrosis in rats.7 Similar beneficial effects were observed by Santiago et al in a transgenic (TG(A1–7)3292) rat model with increased circulating levels of Ang–(1–7) subjected to DOCA-salt treatment. However, in this study, cardioprotection was accomplished in a background of reduced blood pressure (BP), raising the question of whether Ang–(1–7) beneficial effect was a consequence of attenuated BP. Here, we investigated this critical question using a combination of in vivo measurements of cardiac function and molecular biology to evaluate signaling pathways activated in TG rats with chronic increase in plasmatic Ang–(1–7) treated with DOCA.

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Current address for R.d.F.L.: Department of Physiology and Pharmacology, Faculty of Medicine, Universidade Federal do Ceará, Fortaleza, Ceará, Brazil.

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Correspondence to Silvia Guatimosim, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, Pampulha, Belo Horizonte, Minas Gerais CEP 31270-901, Brazil. E-mail guatimosim@icb.ufmg.br

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Detailed Methods are available in the online-only Data Supplement.

Results
TG and Sprague-Dawley (SD) rats presented similar systolic BP before treatment (Figure 1A). However, there were some echocardiographic parameters in TG that differed from those in SD. TG rats presented reduced LV internal dimension (at diastole), cardiac output, stroke volume, end-diastolic and systolic LV volumes, when compared with SD (Table S1 in the online-only Data Supplement). These changes are in keeping with the reduced body weight of TG rats when compared with SD (body weight 481.8±8.2 g, n=11 SD versus 380.5±5.7 g, n=10 TG; P<0.05). Despite the LV volume changes noted, TG and SD rats presented comparable ejection fraction. After 6 weeks, systolic BP was greater in DOCA rats when compared with SD (Figure 1A). The increased BP in SD-DOCA correlated with significant changes in echocardiographic parameters (Table S1), which included decreases in cardiac output and chamber diameter, and a marked increase in LV mass. Moreover, DOCA rats showed decreases in stroke, end-systolic, and end-diastolic volumes. Despite these changes, DOCA-rats presented enhanced ejection fraction. In addition, hearts from SD-DOCA presented global abnormalities in systolic function, as seen by the increase in radial strain rate (Figure 1C), as well as reduced early peak diastolic to peak atrial velocity (E/A) ratio (Figure 1B), which is an indicator of diastolic dysfunction. Thus, after 6 weeks SD-DOCA rats developed concentric LVH and diastolic dysfunction.

Two weeks after the beginning of DOCA treatment, TG rats became hypertensive and remained hypertensive until the end of treatment period (Figure 1A). Comparatively, the DOCA-induced rise in BP was significantly attenuated in TG rats versus SD, as previously described.8 TG-DOCA rats presented significant increases in LV posterior wall and interventricular septal thickness, at systole, when compared with TG (Table S1). All remaining parameters were similar between TG and TG-DOCA. In general, most cardiac changes observed in SD-DOCA were prevented or attenuated in TG-DOCA. In addition, TG-DOCA rats did not show changes in E/A ratio or in radial strain rate (Figure 1B and 1C). Thus, echocardiography data clearly show that lifetime increase in plasmatic carriage of diastolic dysfunction. Hence, after 6 weeks SD-DOCA correlated with significant changes in echocardiographic parameters (Table S1), which included decreases in cardiac output and chamber diameter, and a marked increase in LV mass. Moreover, DOCA rats showed decreases in stroke, end-systolic, and end-diastolic volumes. Despite these changes, DOCA-rats presented enhanced ejection fraction. In addition, hearts from SD-DOCA presented global abnormalities in systolic function, as seen by the increase in radial strain rate (Figure 1C), as well as reduced early peak diastolic to peak atrial velocity (E/A) ratio (Figure 1B), which is an indicator of diastolic dysfunction. Thus, after 6 weeks SD-DOCA rats developed concentric LVH and diastolic dysfunction.

We then investigated potential molecular pathways underlying Ang-(1–7) cardiac protection. To better correlate cellular data with in vivo findings, we used freshly isolated myocytes from LV. Figure 1D shows significant upregulation of hypertrophic markers, atrial natriuretic peptide and β-myosin heavy chain in SD-DOCA myocytes, an effect that was attenuated in LV myocytes from TG-DOCA. In addition, we assessed protein levels of angiotensin-converting enzyme (ACE) 2 and receptor Mas. In TG-DOCA cardiomyocytes, expression of ACE2, the main enzyme responsible for Ang-(1–7) synthesis in the heart,7 is increased when compared with SD or TG (Figure S1A). Yet, expression of Mas was found increased only in SD-DOCA myocytes (Figure S1B).

Ca2+ dysfunction is a major feature in myocyte pathology and alterations in Ca2+ handling proteins have the potential to delay relaxation.10 To determine whether myocytes from SD-DOCA rats present changes in intracellular Ca2+ ([Ca2+]i), we examined Ca2+ levels in electrically stimulated Fluo-4/AM-loaded cells. Figure S2A and S2B show that SD-DOCA cardiomyocytes developed smaller [Ca2+]i transients than SD. In striking contrast, TG-DOCA myocytes presented enhanced Ca2+ transient magnitude. In cardiomyocytes, sarcoplasmic reticulum Ca2+-ATPases (SERCA2) are responsible for the rapid reuptake of cytosolic Ca2+. Therefore, we hypothesized that the reduced magnitude of the [Ca2+]i transient in SD-DOCA may be caused by alterations in SERCA2. Corroborating this assumption, DOCA myocytes presented decreased SERCA2 levels when compared with SD (Figure S2C). TG-DOCA SERCA2 levels were comparable with controls. SERCA activity is regulated by phospholamban, whereas dephosphorylated phospholamban inhibits SERCA, phosphorylation of phospholamban at Thr17 reverses this inhibition.11 Figure S2D shows that SD-DOCA cells presented reduced phospholamban Thr17 phosphorylation. Opposite results were observed in TG-DOCA myocytes. The status of phospholamban phosphorylation is in part dependent on the activity of type 1 phosphatase (PP1).12 Although PP1 levels were significantly increased in cardiomyocytes from SD-DOCA, its expression was reduced in LV-DOCA (Figure S2E). We next assessed protein kinase Cα (PKCα) levels. PKCα directly phosphorylates protein phosphatase inhibitor-1, thus increasing PP1 activity.13 PKCα expression was elevated in SD-DOCA myocytes, a pattern that was not seen in TG-DOCA (Figure S2F). Figure S3A shows that

Figure 1. Transgenic (TG) rats are protected from deoxycorticosterone acetate (DOCA)-induced cardiac remodeling. A, Systolic blood pressure (SBP) obtained in Sprague-Dawley (SD) and TG after DOCA treatment for 6 weeks. *P<0.05 versus SD and TG. #P<0.05 versus SD-DOCA. B, E/A ratio was significantly decreased in SD-DOCA rats. C, SD-DOCA rats showed significantly increased averaged radial strain rate. In B and C, *P<0.05 versus other groups; n=number of rats. D, Upregulation of atrial natriuretic peptide (ANP) and β-myosin heavy chain (β-MHC) transcripts is seen in DOCA cardiomyocytes. Bar graph shows data from at least 5 independent experiments. In D, *P<0.05 versus other groups. #P<0.05 versus SD and SD-DOCA.
extracellular signal–regulated kinase, a key modulator of hypertrophic programming, is more activated in cardiomyocytes from SD-DOCA. This finding is consistent with the ability of Ang-(1–7) to attenuate MR-induced hypertrophy. The serine/threonine kinase Akt is known as a regulator of myocyte survival.14 Figure S3B shows that TG myocytes presented increased phosphorylation of Akt (pAkt), which remains activated in response to DOCA. Conversely, reduced pAkt was found in SD-DOCA myocytes. Altogether, these results demonstrate the profound cardiomyocyte remodeling that occurs in DOCA myocytes. Notably, this signaling profile is modulated in TG-DOCA, indicating that Ang-(1–7)–induced protection against diastolic dysfunction is mediated at the myocyte level, and occurs independently from moderately high BP levels found in TG-DOCA rats. These findings raised the critical question of whether Ang-(1–7) protective signaling was a result of attenuated BP observed in TG-DOCA versus SD-DOCA rats. To address this question, SD rats were subjected to 2 distinct protocols of DOCA treatment to maintain rats at similar BP. In the first protocol, rats received normal DOCA load and BP was kept at the TG-DOCA level by giving hydralazine, an antihypertensive agent (DOCA-Hydra). In the second protocol, rats received reduced amount of DOCA (SD-low-DOCA) to control BP. Figure 2A shows that using either protocol we could efficiently maintain BP in SD-DOCA-Hydra and SD-low-DOCA rats to levels that were similar to TG-DOCA.

Our first set of analyses involved the comparison of echocardiography values obtained from SD rats submitted to each DOCA treatment (Table S2). Under conditions of moderately high BP (≈145 mmHg), both SD-DOCA-Hydra and SD-low-DOCA displayed significant alterations in echocardiography parameters when compared with SD rats. However, the magnitude of LV remodeling was greater in SD-DOCA-Hydra than in SD-low-DOCA rats, confirming a predominant effect of DOCA load in the cardiac alterations observed in this model. Overall, most cardiac parameters were similar between SD-DOCA-Hydra and SD-DOCA except for changes in cardiac output, stroke volume, and end-diastolic LV volume that were more pronounced in SD-DOCA. Nevertheless, despite variations in the magnitude of cardiac remodeling between experimental groups, all SD rats receiving DOCA exhibited diastolic dysfunction (Figure 2B).

Next, we compared echocardiography parameters between SD-DOCA-Hydra and TG-DOCA. Representative images are displayed in Figure 2C. Under conditions of similar BP, the increase in cardiac mass and ejection fraction were more pronounced in SD-DOCA-Hydra than in TG-DOCA. Moreover, significant alterations in chamber dimension, fractional shortening, and E/A ratio were seen only in SD-DOCA-Hydra (Table; Figure 2B) supporting the notion that Ang-(1–7) beneficial effects against DOCA-induced remodeling occur independently from the attenuation in BP.

Next, we investigated the signaling pathways activated in BP-controlled DOCA rats. Under conditions of similar BP, enhanced Ca2+ release was observed in cardiomyocytes from all groups subjected to DOCA treatment (Figure 3A and 3B). Thus, in opposition to data obtained from SD-DOCA, DOCA-Hydra cardiomyocytes did not show reduced Ca2+ levels. The enhanced Ca2+ transient amplitude in DOCA-Hydra cardiomyocytes occurred in a context of reduced SERCA2 protein (Figure 3C) and increased phospholamban Thr17 levels (Figure 3D). Likewise, TG-DOCA myocytes presented increased phospholamban Thr17 phosphorylation, but normal SERCA levels. In addition, DOCA-Hydra cardiomyocytes showed increased PPI, PKCα (Figure 3E and 3F), and phosphorylation of extracellular signal–regulated kinase (Figure S4A). DOCA-Hydra myocytes also presented increased pAkt when compared with SD (Figure S4B). Yet, TG-DOCA pAkt levels were even higher. These cellular data show that DOCA-Hydra rats, maintained under conditions of moderate hypertension, developed a cellular remodeling that resembles in many aspects the profile activated in severely hypertensive SD-DOCA rats.

Our next goal was to compare TG-DOCA with rats that received the half amount of DOCA to control BP. When SD-low-DOCA rats were compared with TG-DOCA, echocardiographic values were generally similar, except for a decrease in chamber dimension (at diastole) that was greater in TG-DOCA (Table). Considering the fact that TG-DOCA rats received almost twice the amount of DOCA given to SD-low-DOCA, this finding once again is in line with our main hypothesis that Ang-(1–7) provides beneficial effects that are independent of its pressor effects. This hypothesis is furthermore reinforced by the observation that SD-low-DOCA rats present a decrease in E/A ratio (Figure 2B) and alterations in both longitudinal and

Figure 2. Blood pressure independent cardioprotection induced by chronic increase in angiotensin (Ang)-(1–7) in deoxycorticosterone acetate (DOCA) rats. A, Similar systolic blood pressure (SBP) levels were imposed in Sprague-Dawley (SD), DOCA-Hydralazine, SD-low-DOCA, and transgenic (TG)-DOCA rats. n=number of rats. *P<0.05 versus SD. B, E/A ratio was significantly reduced in all SD rats receiving DOCA. *P<0.05 versus SD and TG-DOCA. C, Representative M-mode images. LVID indicates left ventricular internal dimension at systole (s) or diastole (d).
radial parameters (Figure S5A; Table S3) that were not seen in TG-DOCA. Likewise, DOCA-Hydra rats presented global changes in contractility. Supporting this finding, upregulation of atrial natriuretic peptide and β-myosin heavy chain transcripts was observed only in LV cardiomyocytes from SD-DOCA-Hydra and SD-low-DOCA rats (Figure S5B).

**Discussion**

Here, we show that in vivo Ang-(1–7) protective signaling against DOCA-induced diastolic dysfunction occurs independently of its lowering BP effects and is mediated at the cardiomyocyte level by the activation of molecular pathways involved in Ca²⁺ handling, hypertrophy, and cell survival. By dissociating the BP effect of Ang-(1–7), we were capable of showing that BP attenuation in TG-DOCA represents a minor component of Ang-(1–7) beneficial effects. In addition, we show that Ang-(1–7) protective signaling occurs even under conditions of moderately high BP because TG-DOCA rats were hypertensive. Altogether, these findings provide the mechanistic input necessary to develop new strategies for

**Table. Echocardiographic Parameters of DOCA Rats Under Conditions of Similar Blood Pressure**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SD (n=5)</th>
<th>DOCA-Hydra (n=7)</th>
<th>TG-DOCA (n=6)</th>
<th>SD-Low-DOCA (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO, mL/min</td>
<td>76.5±2.94</td>
<td>83.0±3.17</td>
<td>63.4±1.65*</td>
<td>79.8±3.38</td>
</tr>
<tr>
<td>LVID (diastole), mm</td>
<td>8.1±0.08</td>
<td>7.2±0.07†</td>
<td>7.7±0.08*</td>
<td>8.3±0.15†</td>
</tr>
<tr>
<td>LVID (systole), mm</td>
<td>5.1±0.10</td>
<td>4.1±0.17†</td>
<td>4.8±0.07*</td>
<td>4.9±0.07*</td>
</tr>
<tr>
<td>LVFW thickness (systole), mm</td>
<td>2.6±0.04</td>
<td>3.2±0.04†</td>
<td>3.0±0.05†</td>
<td>2.9±0.04†</td>
</tr>
<tr>
<td>LVFW thickness (diastole), mm</td>
<td>1.8±0.05</td>
<td>2.1±0.04†</td>
<td>1.9±0.02*</td>
<td>1.8±0.04*</td>
</tr>
<tr>
<td>IVS (systole), mm</td>
<td>2.5±0.04</td>
<td>3.0±0.09†</td>
<td>2.9±0.06</td>
<td>2.7±0.03</td>
</tr>
<tr>
<td>IVS (diastole), mm</td>
<td>1.5±0.02</td>
<td>2.0±0.02†</td>
<td>1.7±0.02*</td>
<td>1.7±0.03†</td>
</tr>
<tr>
<td>LV EF, %</td>
<td>61.5±1.06</td>
<td>79.8±1.08†</td>
<td>69.0±1.44†</td>
<td>74.3±1.96†</td>
</tr>
<tr>
<td>LV FS, %</td>
<td>34.9±0.64</td>
<td>50.2±1.18†</td>
<td>39.9±1.16*</td>
<td>44.8±1.78†</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>329±5.00</td>
<td>333±9.76</td>
<td>337±9.82</td>
<td>333±9.60</td>
</tr>
</tbody>
</table>

CO indicates cardiac output; DOCA, deoxycorticosterone acetate; EF, ejection fraction; FS, fractional shortening; IVS, interventricular septal thickness; LVID, left ventricular internal dimension; LVFW, left ventricular posterior wall; n, number of rats; SD, Sprague-Dawley; and TG, transgenic. *P<0.05 vs SD-DOCA-Hydra. †P<0.05 vs SD. ‡P<0.05 vs TG-DOCA.

**Figure 3.** Under conditions of similar blood pressure (BP), deoxycorticosterone acetate (DOCA) differentially modulated cardiomyocyte signaling in Sprague-Dawley (SD) and transgenic (TG) rats. A, Sample Ca²⁺ transient. B, Bar graph representing peak Ca²⁺ transient amplitude (F/F₀). n=number of cells. C–F (Top), Representative Western blot. Bottom, Bar graph showing the relative expression of SERCA2, phosphorylated phospholamban (pPLN) Thr17, type 1 phosphatase (PP1), and protein kinase Cα (PKCα) in LV myocytes. n=number of samples. *P<0.05 versus other groups. †P<0.05 when compared with SD. a.u. indicates arbitrary units; and GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
clinical use of Ang-(1–7) alone or in combination with other drugs in patients with diastolic heart failure (HF).

Clinical studies have demonstrated the importance of MR antagonists for the prognostic improvement of patients with chronic HF. Preclinical data have shown strong evidence that Ang-(1–7) effectively prevents cardiac remodeling induced by DOCA-salt treatment, suggesting that Ang-(1–7) could be an important therapeutic strategy to reduce pathology associated to conditions of increased MR activation. In addition, previous study has linked the beneficial effects of MR blocker therapy to increased cardiac ACE2 activity raising the possibility that increases in Ang-(1–7) formation could contribute to the beneficial effects of MR antagonism. Here, we also provide evidence that cardioprotective signaling against diastolic dysfunction correlates with enhanced ACE2, by showing the increase in ACE2 expression in TG-DOCA myocytes.

Diastolic dysfunction is a common entity in hypertensive patients. In our model, activation of MR leads to hypertensive disease and diastolic dysfunction in SD rats. When BP of SD-DOCA rats was maintained constant by the administration of hydralazine or by reducing the amount of DOCA, the magnitude of LVH was attenuated, but it was still significant. In addition, both diastolic dysfunction and abnormalities in global contractility were evident in hearts from DOCA-Hydra and SD-low-DOCA. Thus, we conclude that both BP level and DOCA load contribute to the magnitude of cardiac dysfunction induced by MR activation. This observation is consistent with a previous study showing that pharmacological control of BP is associated to a decrease in LV mass and supported by clinical data showing that tighter control of systolic BP was associated with a reduction in development of LV hypertrophy. However, it is clear that BP-independent actions of DOCA exert a prominent role during the development of diastolic dysfunction with lower degree of remodeling found in SD-low-DOCA when compared with DOCA-Hydra.

In TG rats, progression of hypertensive disease was slowed because the development of LVH was significantly attenuated and both diastolic dysfunction and global abnormalities in systolic function were prevented. The fact that these beneficial effects occurred independently of Ang-(1–7) effects on BP may indicate a mechanism of considerable clinical importance. For instance, it shows that even under moderately high BP conditions, Ang-(1–7) is capable of activating specific signaling pathways in cardiomyocytes that are involved in attenuation of LVH thus delaying the onset of diastolic dysfunction. The fact that LVH is an independent risk factor for the development of HF reinforces the importance of this finding. In addition, others have demonstrated that Ang-(1–7) prevents extracellular matrix deposition, a key event in the transition from LVH to symptomatic HF that may also contribute to Ang-(1–7) protection in TG-DOCA.

Apart from hemodynamic factors, humoral factors, such as endothelin, and sympathetic activation also contribute to the cardiovascular remodeling observed in DOCA rats. This knowledge further reinforces the idea that Ang-(1–7) exerts beneficial effects even under conditions that involve complex activation of different signaling pathways.

Santiago et al have previously reported an increase in cardiac Ang-(1–7) levels in TG-DOCA rats, a finding that is further supported by our data showing enhanced ACE2 expression in TG-DOCA myocytes. The increase in cardiac ACE2/Ang-(1–7) provides the basis for direct local protective effects of Ang-(1–7) in cardiac cells from TG-DOCA, yet the mechanisms mediating this phenomenon remain unclear.

Our data suggest that in vivo Ang-(1–7) acts directly in ventricular cells modulating signaling pathways that are activated during the progression from hypertensive disease to diastolic dysfunction. At the cardiomyocyte level, the signaling profile activated in DOCA-Hydra rats is distinct from that seen in TG-DOCA. This knowledge implies that the attenuation of BP by Ang-(1–7) represents a minor component of Ang-(1–7) protective actions. Although antihypertensive effects of Ang-(1–7) have been previously reported, our findings show a lack of effect of Ang-(1–7)/Mas receptor blockers on BP levels. Further studies will be required to elucidate under what conditions Ang-(1–7) affects or does not affect BP.

Impaired Ca2+ homeostasis is a key feature in the transition from compensatory hypertrophy to HF. SD-DOCA myocytes present abnormal Ca2+ handling caused, at least in part, by reduced SERCA2 activity. This reduction in SERCA2 activity reduces the speed of removal of Ca2+ from the cytoplasm to the sarcoplasmic reticulum, thereby contributing to diastolic dysfunction. Likewise, SD-DOCA-Hydra myocytes presented reduced SERCA2. Yet, phospholamban phosphorylation at Thr17 and Ca2+ transient amplitude were elevated in comparison with SD, indicating that the increase in phospholamban phosphorylation compensated for reduced SERCA2 in DOCA-Hydra myocytes. In hypertensive TG-DOCA rats, we observe the ability of Ang-(1–7) to prevent DOCA-induced SERCA2 downregulation. This is an important aspect of in vivo Ang-(1–7) signaling because other groups have demonstrated a critical role of SERCA in attenuating the transition to HF in pressure-overloaded mice.

It is worth mentioning the fact that increased phosphorylated phospholamban at Thr17 occurs in cardiomyocytes from SD-DOCA-Hydra rats, and its phosphorylation is maintained in TG-DOCA rats, indicating that DOCA-induced phosphorylation of phospholamban is preserved in conditions of increased Ang-(1–7) signaling. Likewise, we have previously demonstrated in vitro that aldosterone treatment of cardiomyocytes leads to an increase in phosphorylated phospholamban that is maintained in the presence of Ang-(1–7). Contrary to what is seen with phospholamban, most of the cellular changes induced by MR activation are antagonized by Ang-(1–7). Accordingly, previous studies have shown the ability of Ang-(1–7) to antagonize the increase in extracellular signal–regulated kinase phosphorylation induced by Ang II. Another key feature of cardiomyocytes from TG-DOCA rats is the decrease in PPI levels. PPI expression is upregulated in failing hearts and increases in its activity can account for reduced sarcoplasmic reticulum Ca2+ uptake and therefore reduced ventricular function.

PKCα is known to phosphorylate titin, the myofilament protein responsible for cardiomyocyte passive tension. Alteration in titin phosphorylation represents an important mechanism in the development of diastolic dysfunction.
Elevation of PKCα was observed in all SD-DOCA receiving rats, including SD-low-DOCA (data not shown), but it was absent in TG-DOCA, which may explain the improvement in diastolic dysfunction in this model. Consistent with these results, Mori et al32 showed that Ang-(1–7) infusion prevented PKCα elevation in db/db mice.

Of all signaling pathways investigated, only Akt was activated in TG rats. The fact that Akt represents an important survival pathway36 is consistent with the cardioprotective role of Ang-(1–7). Supporting this assumption, we and others have previously shown that Ang-(1–7) leads to increased pAkt in the heart.7,38 Thus, Ang-(1–7) protective signaling against diastolic dysfunction occurs independently of its lowering BP effects and is mediated, at least in part, at the myocyte level by the activation of key signaling pathways.

Perspectives

Ang-(1–7) is a biologically active end product of the renin–angiotensin system, with great potential for therapeutic use. The finding that Ang-(1–7), independently from its BP lowering effects, activates signaling pathways in cardiomyocytes that prevent the transition from LVH to diastolic dysfunction is potentially important because it provides the knowledge for the use of Ang-(1–7) alone or in combination with other drugs for the treatment of patients with cardiac disease.

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Disclosures

None.

References


### Novelty and Significance

**What Is New?**

- Angiotensin-(1–7) protective signaling against diastolic dysfunction occurs independently of its lowering blood pressure effects and is mediated, at least in part, at the myocyte level by the activation of signaling pathways involved in Ca2+ regulation, hypertrophic growth, and survival.

**What Is Relevant?**

- This work provides important mechanistic input that could help to develop new therapeutic strategies based on the use of angiotensin-(1–7) alone or in combination with other drugs for patients with hypertensive disease.

### Summary

Angiotensin-(1–7) slows the development of hypertensive disease by preventing the transition from left ventricular hypertrophy to diastolic dysfunction independently from its blood pressure lowering effects.
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Short title: Ang-(1-7) mechanisms against DOCA dysfunction

1Pedro W. Machado de Almeida; 1,3Marcos Barrouin Melo; 1,4Ricardo de Freitas Lima; 1Mariana Gavioli; 1Nivia M. Santiago; 1Leonardo Greco; 1Itamar C.G. Jesus; 1Eduardo Nocchi; 1Amanda Parreira; 1Márcia N.M. Alves; 1Luciana Mitraud; 2Rodrigo Ribeiro Resende; 1,3Maria José Campagnole-Santos; 1,3Robson Augusto Souza dos Santos; 1,3Silvia Guatimosim

Departments of 1Physiology and Biophysics, and 2Biochemistry and Immunology, Institute of Biological Sciences, Universidade Federal de Minas Gerais, CEP31270-901, Belo Horizonte, Minas Gerais, Brazil. 3National Institute of Science and Technology in Nanobiopharmaceutics 4Current address: Department of Physiology and Pharmacology, Faculty of Medicine, Universidade Federal do Ceará, Fortaleza, Ceará, Brazil
Methods

Animals

35 Male Sprague-Dawley (SD) and 37 transgenic rats with lifetime increase in plasmatic Ang-(1-7) (3-4 months old) (TG(A1-7)3292) were obtained from the transgenic animal facilities of the Federal University of Minas Gerais (Hypertension Laboratory). All experimental protocols conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by our institution. Phenotypic data about transgenic rats were already published.¹

DOCA-salt hypertension

Rats were anesthetized and unilaterally nephrectomized. Silicone rubber impregnated with DOCA (50 mg/kg/week; Sigma) was implanted subcutaneously, and rats were offered 0.5% saline/0.2% KCl to drink, during 6 weeks, as previously described by our group.² SD-DOCA-Hydra rats received the same amount of DOCA (50mg/kg/week) plus 10mg/kg/day hydralazine added to the drinking water. Control rats were also uninephrectomized but received a silicone rubber implant without DOCA and were offered tap water to drink. SD-Low-DOCA rats received 30mg/kg/ week during 6 weeks. Systolic arterial pressure (SAP) was evaluated by tail cuff plethysmography (RTBP2000, Kent Scientific), one day before the DOCA implant and every week during treatment.

Echocardiography Analysis. Animals underwent transthoracic echocardiographic examination after 6 weeks of DOCA-Salt treatment. In vivo cardiac morphology and function were assessed noninvasively using a high-frequency, high-resolution echocardiographic system consisting of a VEVO 2100 ultrasound machine equipped with a 16–21MHz bifrequencial transducer (Visual Sonics, Toronto, Canada). The rats were anaesthetized with 5% isoflurane for induction, and placed in supine position on an imaging stage equipped with built-in electrocardiographic electrodes for continuous heart rate monitoring and a heater to maintain the body temperature at 37°C. Anesthesia was sustained via a nose cone with 2.5% isoflurane. Ultrasound gel was applied to the anterior chest that was shaved after anesthesia induction and high-resolution images were obtained in the right and left parasternal long and short axes and apical orientations. Standard B-mode images of the heart and pulsed wave Doppler images of the mitral inflow were acquired. Left ventricular (LV) dimensions and wall thickness were measured at the level of the papillary muscles in left and right parasternal long and short axes during the end systole and end diastole. LV ejection fraction (EF) and fractional shortening (FS) were derived from chamber measurements. All measurements and calculations were done in accordance with the American Society of Echocardiography. The following M-mode measurements were performed: LV internal dimensions at diastole and systole (LVIDd and LVIDs), LV posterior wall dimensions at diastole and systole (LVPWd and LVPWs), and interventricular septal thickness at diastole and systole (IVSd and IVSs). Based on these parameters, end diastolic and end systolic LV volumes (EDLVV and ESLVV), FS, EF, stroke volume (SV), and cardiac output (CO) were calculated. Also, the radial strain from the bidimensional long axis
view of the left ventricle was performed using the Vechostrain software. The following parameters were evaluated: velocity, displacement, strain, and strain rate.

**Real Time PCR.** Real time PCR was used as previously described to verify mRNA levels of ANP and β-MHC.³

**Freshly isolated ventricular myocytes and Ca²⁺ recording**

Adult ventricular myocytes were freshly isolated and stored in DMEM media (Sigma), until they were used (within 1 h), as previously described.⁴ Intracellular Ca²⁺ ([Ca²⁺]i) imaging experiments were performed in Fluo-4 AM loaded-cardiomyocytes⁵. Images were obtained using the ZEISS Meta confocal microscope (Zeiss Germany) from CAPI (Centro de Aquisição e Processamento de Imagens, ICB/UFMG).

**Western blot**

Adult ventricular myocytes were harvested as described above and protein content was quantified according to Bradford protein assay. We used 40-60 µg of protein, and the samples were separated by SDS-PAGE. Antibodies and their sources are as follows: anti-SERCA2 (1:1000 ABR), anti-PKC alpha (1:1000, Millipore), anti-PP1 (1:1000, Santa Cruz), anti-phospho-ERK (1:1000, Cell Signaling), anti-ERK (1:1000, Cell Signaling), anti-PLN Thr¹⁷ (1:1000 Santa Cruz), anti-phospho-AKT Ser¹⁷³ (1:1000, Cell Signaling), anti-AKT (1:1000, Cell signaling), anti-ACE2 (1:1000, Santa Cruz), anti-receptor Mas (1:1000, Alomone) and anti-GAPDH (1:3000, Santa Cruz). Protein expression levels were detected by chemiluminescence reaction (ECL plus, GE Healthcare) and the intensity of them was evaluated by densitometric analysis using ImageQuant LAS 4000 (GE Healthcare). Protein levels were normalized by using anti-GAPDH antibody.

**Statistical Analyses.** Data are presented as mean ± SEM. For comparisons of two variables we used Student's t test and for comparison of more than two variables we used One Way or Two Way ANOVA. The level of significance was set to values of p<0.05.

References


Table S1: Summary of DOCA-induced cardiac changes in SD and TG rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SD (n=5)</th>
<th>SD-DOCA (n=6)</th>
<th>TG CT (n=5)</th>
<th>TG-DOCA (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO (mL/min)</td>
<td>81.35 ± 3.85</td>
<td>52.20 ± 5.01*</td>
<td>63.27 ± 2.75*</td>
<td>60.18 ± 3.78*</td>
</tr>
<tr>
<td>LVID (diastole, mm)</td>
<td>8.16 ± 0.10</td>
<td>6.88 ± 0.28*</td>
<td>7.25 ± 0.15*</td>
<td>7.63 ± 0.17</td>
</tr>
<tr>
<td>LVID (systole, mm)</td>
<td>5.44 ± 0.19</td>
<td>4.21 ± 0.17*</td>
<td>4.76 ± 0.15</td>
<td>4.90 ± 0.17</td>
</tr>
<tr>
<td>LVPW thickness (systole, mm)</td>
<td>2.52 ± 0.08</td>
<td>3.31 ± 0.09*</td>
<td>2.60 ± 0.05‡</td>
<td>3.00 ± 0.03*‡‡</td>
</tr>
<tr>
<td>LVPW thickness (diastole, mm)</td>
<td>1.73 ± 0.03</td>
<td>2.33 ± 0.12*</td>
<td>1.81 ± 0.07‡</td>
<td>1.93 ± 0.02‡</td>
</tr>
<tr>
<td>IVS (systole, mm)</td>
<td>2.46 ± 0.04</td>
<td>3.18 ± 0.13*</td>
<td>2.36 ± 0.11‡</td>
<td>2.86 ± 0.06†</td>
</tr>
<tr>
<td>IVS (diastole, mm)</td>
<td>1.51 ± 0.04</td>
<td>2.12 ± 0.09*</td>
<td>1.54 ± 0.04‡</td>
<td>1.76 ± 0.02*‡</td>
</tr>
<tr>
<td>LV EF (%)</td>
<td>62.60 ± 1.59</td>
<td>78.95 ± 1.06*</td>
<td>64.83 ± 1.53‡</td>
<td>68.30 ± 1.54‡</td>
</tr>
<tr>
<td>LV FS (%)</td>
<td>35.31 ± 1.13</td>
<td>48.80 ± 1.15*</td>
<td>36.69 ± 1.13‡</td>
<td>39.28 ± 1.25‡</td>
</tr>
<tr>
<td>LV SV (μL)</td>
<td>241.6 ± 10.11</td>
<td>171.7 ± 13.22*</td>
<td>182.5 ± 4.24*</td>
<td>174.0 ± 6.94*</td>
</tr>
<tr>
<td>End diastolic LV volume (μL)</td>
<td>388.4 ± 25.09</td>
<td>216.8 ± 14.27*</td>
<td>282.5 ± 11.96*</td>
<td>255.4 ± 11.7*</td>
</tr>
<tr>
<td>End-systolic LV volume (μL)</td>
<td>146.8 ± 15.29</td>
<td>45.15 ± 1.46*</td>
<td>100.0 ± 8.48‡</td>
<td>81.41 ± 6.74*‡</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>336.9 ± 10.79</td>
<td>303.3 ± 10.23</td>
<td>347.5 ± 16.98</td>
<td>344.6 ± 8.56</td>
</tr>
</tbody>
</table>

CO - cardiac output, LVID - left-ventricular internal dimension, LVPW - LV posterior wall, IVS - interventricular septal thickness; EF - Ejection Fraction; FS - Fractional shortening; HR - heart rate. * = p<0.05 versus SD; † = p<0.05 versus TG, ‡ = p<0.05 versus SD-DOCA. n = number of rats.
Table S2. Summary of changes in echocardiography parameters between SD rats following DOCA-treatment maintained or not under similar BP.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SD (n=5)</th>
<th>SD-DOCA (n=6)</th>
<th>SD-Hydra (n=7)</th>
<th>SD-low DOCA (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO (mL/min)</td>
<td>76.52 ± 6.94</td>
<td>52.20 ± 5.01*</td>
<td>83.04 ± 3.17†</td>
<td>79.77 ± 3.38†</td>
</tr>
<tr>
<td>LVID (diastole, mm)</td>
<td>8.15 ± 0.08</td>
<td>6.88 ± 0.28*</td>
<td>7.22 ± 0.07*</td>
<td>8.34 ± 0.15††</td>
</tr>
<tr>
<td>LVID (systole, mm)</td>
<td>5.15 ± 0.10</td>
<td>4.21 ± 0.17*</td>
<td>4.15 ± 0.17*</td>
<td>4.97 ± 0.07††</td>
</tr>
<tr>
<td>LVPW thickness (systole, mm)</td>
<td>2.61 ± 0.04</td>
<td>3.31 ± 0.09*</td>
<td>3.21 ± 0.04*</td>
<td>2.93 ± 0.04††</td>
</tr>
<tr>
<td>LVPW thickness (diastole, mm)</td>
<td>1.81 ± 0.05</td>
<td>2.33 ± 0.12*</td>
<td>2.18 ± 0.04*</td>
<td>1.80 ± 0.04††</td>
</tr>
<tr>
<td>IVS (systole, mm)</td>
<td>2.58 ± 0.04</td>
<td>3.18 ± 0.13*</td>
<td>3.01 ± 0.09*</td>
<td>2.77 ± 0.03</td>
</tr>
<tr>
<td>IVS (diastole, mm)</td>
<td>1.56 ± 0.02</td>
<td>2.12 ± 0.09*</td>
<td>2.02 ± 0.02*</td>
<td>1.77 ± 0.03††</td>
</tr>
<tr>
<td>LV ejection fraction (%)</td>
<td>61.52 ± 1.06</td>
<td>78.95 ± 1.06*</td>
<td>79.83 ± 1.08*</td>
<td>74.31 ± 1.96††</td>
</tr>
<tr>
<td>LV fractional shortening (%)</td>
<td>34.92 ± 0.64</td>
<td>48.80 ± 1.15*</td>
<td>50.27 ± 1.18*</td>
<td>44.80 ± 1.78††</td>
</tr>
<tr>
<td>LV systolic volume (μL)</td>
<td>231.5 ± 17.85</td>
<td>171.7 ± 13.22*</td>
<td>248.6 ± 6.34†</td>
<td>225.9 ± 8.45†</td>
</tr>
<tr>
<td>End diastolic LV volume (μL)</td>
<td>377 ± 29.50</td>
<td>216.8 ± 14.27*</td>
<td>312.8 ± 6.95†</td>
<td>303.9 ± 6.66†</td>
</tr>
<tr>
<td>End systolic LV volume (μL)</td>
<td>145.5 ± 12.61</td>
<td>45.15 ± 1.46*</td>
<td>64.51 ± 4.42*</td>
<td>77.96 ± 5.98†</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>329 ± 5.00</td>
<td>303.3 ± 10.23</td>
<td>333.6 ± 9.76</td>
<td>353.4 ± 9.60†</td>
</tr>
</tbody>
</table>

For comparison purposes, SD-DOCA values were included in this table. CO-cardiac output; LVID- left-ventricular internal dimension; LVPW- LV posterior
wall; IVS- interventricular septal thickness; HR- heart rate. *=p<0.05 versus SD, †=p<0.05 versus SD-DOCA, ‡= p<0.05 versus SD-Hydra. n= number of rats.
Table S3. Ang-(1-7) prevents global changes in myocardial deformation following DOCA-treatment.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SD (n=8)</th>
<th>SD-Hydra (n=7)</th>
<th>TG-DOCA (n=7)</th>
<th>SD-low DOCA (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radial Strain (PK %)</td>
<td>16.79±1.63</td>
<td>29.20±1.16*†</td>
<td>19.52±1.15</td>
<td>24.44±0.59*</td>
</tr>
<tr>
<td>Longitudinal Strain (PK %)</td>
<td>-14.55±1.21</td>
<td>-24.37±2.21*†</td>
<td>-12.60±1.04</td>
<td>-22.39±1.12*†</td>
</tr>
<tr>
<td>Radial Strain rate (PK 1/S)</td>
<td>4.60±0.30</td>
<td>6.77±0.27*†</td>
<td>4.56±0.23</td>
<td>6.46±0.39*†</td>
</tr>
<tr>
<td>Longitudinal Strain rate (PK 1/S)</td>
<td>-3.53±0.19</td>
<td>-5.80±0.33*†</td>
<td>-3.62±0.27</td>
<td>-5.63±0.51*†</td>
</tr>
</tbody>
</table>

n= number of rats analysed in each experimental group. *=p<0.05 versus SD, †= p<0.05 versus TG-DOCA.
Figure S1: Differential expression of ACE2 and receptor Mas in myocytes from SD and TG following DOCA-treatment. A-B. Top, representative western-blot. Bottom, ACE2 and Mas expression levels in left-ventricular myocytes from each experimental group. n= number of samples analysed. *=p<0.05 when compared to the other groups.
FIGURE S2
Figure S2: DOCA-induced changes in signaling profile are prevented in TG rats. A. Sample Ca\(^{2+}\) transient from LV-myocytes. B. Bar graph representing peak Ca\(^{2+}\) transient magnitude (F/F\(_0\)). n= number of cells. C-F. Left, representative western-blot. Right, bar graph shows the mean density of the bands in arbitrary units (a.u). n = numbers of cardiomyocyte samples. *= p <0.05 versus other groups.
FIGURE S3

Figure S3: Opposing regulation of ERK and Akt phosphorylation in cardiomyocytes from SD and TG treated with DOCA. Top, representative western-blots of ERK (A) and Akt (B) phosphorylation. Bottom, bar graph representing the mean density of pERK (A) and pAkt (B) bands in arbitrary units (a.u.). *= p <0.05 versus other groups. # = p<0.05 versus SD and SD-DOCA. n= numbers of cardiomyocyte samples.
Figure S4: Protective signaling induced in left-ventricular myocytes from TG(A1-7)3292 occurs independently of changes in BP. A-B. Top, Representative western-blot. Bottom, bar graph showing the relative expression of pERK (A) and pAkt (B) levels in LV-myocytes from SD, DOCA-Hydra and TG-DOCA rats. n= number of myocyte samples.* = p <0.05 versus other groups.
**FIGURE S5**

Figure S5: Under conditions of similar BP pathological remodeling is prevented in TG rats with chronic increase in Ang-(1-7). **A.** Representative tridimensional images of strain. **B.** Real-time PCR showing significant upregulation of ANP and β-MHC transcripts only in cardiomyocytes from SD-low DOCA and DOCA-Hydra rats. The bar graph shows data from at least 5 independent experiments. *= p <0.05 versus SD and TG-DOCA. #$=p<0.05$ when compared to the other groups.