



**Improved cardiovascular autonomic modulation in transgenic rats expressing an Ang-(1-7)-producing fusion protein**

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**Improved cardiovascular autonomic modulation in transgenic rats  
expressing an Ang-(1-7)-producing fusion protein**

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**Running title:** Ang-(1-7) and cardiovascular autonomic control

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**Abstract**

Angiotensin-(1-7) counterbalances Angiotensin II cardiovascular effects. However, it has yet to be determined how cardiovascular autonomic modulation may be affected by chronic and acute elevation of Ang-(1-7). Hemodynamics and cardiovascular autonomic profile were evaluated in male Sprague-Dawley (SD) and transgenic rats (TGR) overexpressing Ang-(1-7) [TGR(A1-7)3292]. Blood pressure (BP) was directly measured while cardiovascular autonomic modulation was evaluated by spectral analysis. TGR received A-779 or vehicle and SD rats received Ang-(1-7) or vehicle and were monitored for 5 hours after *i.v.* administration. In another set of experiments with TGR, A-779 was infused for 7 days using osmotic mini pumps. Although at baseline no differences were observed, acute administration of A-779 in TGR produced a marked long lasting increase in BP accompanied by increased BP variability (BPV) and sympathetic modulation to the vessels. Likewise, chronic administration of A-779 with osmotic mini pumps in TGR increased heart rate, sympathovagal balance, BPV and sympathetic modulation to the vessels. Administration of Ang-(1-7) to SD rats increased HRV values in 88% accompanied by 8% of vagal modulation increase and 18% of mean BP reduction. These results show that both acute and chronic alteration in the Ang-(1-7)-Mas receptor axis may lead to important changes in the autonomic control of circulation, impacting either sympathetic and/or parasympathetic systems.

**Keywords:** Renin-angiotensin system, angiotensin-(1-7), A-779, blood pressure, autonomic nervous system, heart rate variability, spectral analysis.

## Introduction

The renin-angiotensin system (RAS) is a key regulatory mechanism of the renal, cardiovascular and autonomic nervous systems (Baltatu et al. 2011). The discovery and characterization of Ang-(1-7) and its receptor *Mas* is one of the most significant recent changes in the understanding of the system, particularly the mechanisms linked to this axis, which counterbalance most of the deleterious effects of Ang II (Ferrario 2011; Oudit et al. 2003; Reudelhuber 2011; Santos et al. 2008; Santos and Andrade 2014).

The beneficial biological effects of Ang-(1-7) described in the last two decades include vasodilation, antiproliferative effects on smooth muscle cells (Freeman et al. 1996), increased baroreflex sensitivity (Campagnole-Santos et al. 1992; Oliveira et al. 1996), decreased oxidative stress (Fraga-Silva et al. 2008), and improved glucose and lipid metabolism (Santos et al. 2010). In a recent study conducted by our group, using a rat model of genetic hypertension, we observed a decrease in blood pressure and an improvement in autonomic modulation of the cardiovascular system after chronic treatment with an oral formulation of Ang-(1-7) (Bertagnolli et al. 2014).

The autonomic nervous system seems to be a key mechanism in the cardiovascular responses to Ang-(1-7), since intracerebroventricular injection or microinjections of the peptide in the nucleus of the solitary tract facilitates the baroreflex control of heart rate as opposed to the Ang II-induced effects (Campagnole-Santos et al. 1988). Moreover, when these peptides were injected in the rostral ventrolateral medulla, no effects on baroreflex were observed. However, when injected in the caudal portion of the ventral lateral medulla, both

the bradycardic and the tachycardic components of the baroreflex were affected (Alzamora et al. 2006). Recently, it has been reported that peripheral or central injection of Ang-(1-7) can modulate the cardiac component of acute stress response, which is characterized by a sympathetic nervous system activation, reinforcing the interaction between RAS and the autonomic nervous system (Martins Lima et al. 2013).

Unlike the aforementioned studies, which were based on the acute administration of Ang-(1-7), investigations using transgenic rats with a life-long overexpression of a fusion protein which produces Ang-(1-7) model (TGR(A1-7)3292) did not find any differences in blood pressure when compared to age-matched control SD rats at baseline. Nevertheless, they did show alterations in heart rate and regional increase in blood flow regulation (Botelho-Santos et al. 2007; Santos et al. 2004), suggesting that the chronic alteration of the Ang-(1-7)-Mas receptor axis may promote compensatory physiological adjustments which would then impose different autonomic balance conditions. Based on these findings, we hypothesize whether a life-long increase in Ang-(1-7), as observed in the TGR(A1-7)3292 model, generates changes in the autonomic modulation to the heart and the vessels.

## Methods

All the procedures adopted in this study were in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy Press 2011), and the guidelines from the Canadian Council on Animal Care (available

at [www.ccac.ca](http://www.ccac.ca)). This study was approved by the local ethics committee (CEUA/IC-FUC) under the protocol number UP: 2546/2009.

### *Animals*

Fifteen week old transgenic rats overexpressing a fusion protein which produces Ang-(1-7) [TGR(A1-7)3292], and age-matched Hannover Sprague-Dawley rats, weighing 250g, were obtained from the Biological Sciences Institute of Federal University of Minas Gerais (ICB-UFMG, Brazil). Rats were kept under standard and controlled environment conditions of temperature (22°C-24°C), light and dark cycles (12 hours each, 06:00-18:00) with standard chow and water *ad libitum*.

### *Groups and protocols*

Due to its own genetic characteristics, transgenic rats [TGR(A1-7)3292] (TGR), presenting a chronic increase of 2.5-fold in Ang-(1-7) plasmatic concentration (Botelho-Santos et al. 2007) were enrolled to mimic the chronic state of Ang-(1-7) elevation in this protocol, while Sprague Dawley Hannover rats (SD) were used as control (n = 8/group).

SD rats received in *bolus* injections of Ang-(1-7) or a saline solution, while TGR received in *bolus* injection of saline or A-779, a Mas receptor antagonist. For these acute experiments, rats were monitored for 5 hours after the injection.

In another set of experiment, in order to induce the blockade of Ang-(1-7)-Mas receptor axis, the Mas antagonist A-779 was released through osmotic

mini-pumps for 7 days in the TGR (TGR op) (n = 8/group) before hemodynamic, and autonomic control evaluations.

#### *Blockade of Mas receptors with A-779*

Osmotic mini pumps (Alzet®, Cupertino, CA - Micropump model 2001) containing A-779 (200µl – 12.5 nmol/Kg) were implanted in transgenic rats (TRG op) under anesthesia (50 mg/kg, i.p., ketamine and 10 mg/kg, i.p., xylazine). The mini pumps were kept for 7 days, at an infusion rate of 1 µL/hour, when the hemodynamic evaluation was performed (Braga et al. 2002; Britto et al. 1997).

#### *Ang-(1-7) and A-779 in bolus injections*

Both SD and TGR groups underwent the acute protocol and were randomly assigned to different injections. SD rats received *in bolus* intravenous injections of Ang-(1-7) (2.5 nmol/kg) diluted in a 0.9% NaCl solution or of saline solution (vehicle 0.9% NaCl). TGR were injected with A-779 (12.5 nmol/kg) diluted in a 0.9% NaCl solution or with saline solution (vehicle 0.9% NaCl) (Braga et al. 2002; Britto et al. 1997). After injections, animals were monitored for 5 consecutive hours.

#### *Hemodynamic assessment*

Rats were anaesthetized (50 mg/kg, i.p., ketamine and 10 mg/kg, i.p., xylazine) and catheters were inserted into the femoral artery and vein for direct measurement of blood pressure and drug administration, respectively. Conscious rats were studied 24h after catheter placement and were allowed to

move freely during the experiments. The arterial cannula was connected to a strain gauge transducer (Blood Pressure XDCR; Kent Scientific, Litchfield, CT, USA) and blood pressure signals were recorded by a microcomputer equipped with an analogue-to-digital converter board (Windaq; 2 kHz sampling frequency; Dataq Instruments, Springfield, OH, USA). TGR and SD groups, which received *in bolus* injections, were recorded for 5 hours after the injections of Ang-(1-7) and A-779. Continuous blood pressure signals of TGRop rats were recorded for 30 minutes. All the sampled data were analyzed on a beat-to-beat basis to quantify changes in mean blood pressure and heart rate (Moraes-Silva 2010; Moreira et al. 2013).

#### *Cardiovascular autonomic control evaluation*

From time series of heart rate (tachogram) and systolic arterial pressure (systogram), obtained from the direct hemodynamic measurements, stationary sequences of 300 beats were selected for the spectral analysis using the autoregressive method (Malliani et al. 1991; Pagani et al. 1997; Parati et al. 1995). The oscillatory components of the tachogram and systogram were analyzed in three different frequency bands with distinct physiological meanings: HF (High Frequency: 0.75-3.0 Hz); LF (Low Frequency: 0.25-0.75 Hz), and VLF (Very Low Frequency: 0.0-0.25 Hz). Heart rate variability (HRV) and systolic arterial pressure (SAP) variability were obtained from the total power spectrum. The LF component extracted from HRV and SAP variability has been found to be related to sympathetic cardiac and vascular modulation, respectively, while the HF component of HRV would reflect vagal cardiac

modulation. The ratio between LF and HF components represents the cardiac sympathovagal balance. VLF power of blood pressure variability in rats is related to RAS modulation under blood pressure. The relationship between the LF component of HRV and SAP variability corresponds to the spontaneous baroreflex sensitivity (alpha index) (Fazan et al. 2005).

### *Statistical analysis*

Data were presented as mean  $\pm$  SD. Comparisons of baseline characteristics were performed using Student *t* test. Results from *in bolus* injections were compared using Two-way ANOVA, followed by Tukey's post-hoc test. Data obtained from the TGRop rats were compared with One-way ANOVA followed by Tukey's post-hoc test. The GraphPad Prism 6.0 for Windows (GraphPad Software, San Diego, CA, United States) software was used for all tests undertaken. The significance level was established at  $p < 0.05$ .

### **Results**

As seen in Table 1, no differences between SD and TGR groups were found in blood pressure, heart rate, and autonomic modulation at baseline conditions.

#### *Cardiovascular effects of acute Ang-(1-7) administration in SD rats*

Figure 1 depicts the effects of saline injection and Ang-(1-7) over time in SD rats. We observed an increase in heart rate variability with Ang-(1-7) four hours after injection, accompanied by augmented cardiac parasympathetic modulation, expressed by the high frequency component (HF). Moreover, a

reduction in systolic and diastolic blood pressure after Ang-(1-7) injection was found when compared to the vehicle. Control rats receiving acute injection of saline solution and A-779 did not present significant alterations neither in hemodynamics nor in autonomic control. The acute effects of Ang-(1-7) were blocked by co-administration of A-779 (data not shown).

In addition, the concomitant administration of Ang-(1-7) and A-779 in SD (control) animals promoted punctual alterations in blood pressure variability parameters 1 hour after injection, returning to baseline at the 3<sup>rd</sup> hour. Also, an increase in sympathovagal balance was observed at the 5<sup>th</sup> hour, indicating sympathetic predominance (data not shown).

#### *Cardiovascular effects of acute blockade of Ang-(1-7) in TGR by A-779*

Acute administration of A-779 produced pronounced cardiovascular and autonomic effects in the TGR group (Figure 2). We found a significant increase in heart rate at the 3<sup>rd</sup> hour of injection without any alterations in heart rate variability parameters. Nevertheless, expressive changes in blood pressure variability parameters were observed, as depicted in Figure 2. More importantly, A-779 promoted a dramatic elevation in systolic and diastolic blood pressure, as well as in the low frequency component of blood pressure variability. These changes were observed from the 2<sup>nd</sup> to the 4<sup>th</sup> hour of A-779 injection. Values returned to baseline values at the 5<sup>th</sup> hour.

*Cardiovascular effects of chronic blockade of Ang-(1-7) in TGR*

After 7 days of A-779 infusion, the TRGop (osmotic pump) group presented significant increases in heart rate, blood pressure, sympathovagal balance, blood pressure variability, and LF component of blood pressure variability (Figure 3).

**Discussion**

This is the first study demonstrating that both acute and chronic interventions in the Ang-(1-7)-Mas axis may modify cardiovascular responses mediated by changes in the modulation of autonomic nervous system activity. In this study, we used TGR(A1-7)3292 as a model for a chronic increase in Ang-(1-7). Sampaio et al. have shown that Ang-(1-7) produces a significant augmentation of cardiac output and stroke volume in Wistar rats (Sampaio et al. 2003). Accordingly, a similar increase was also observed in TGR (A1-7)3292 rats (Botelho Santos et al. 2007). These rats also presented a slight increase in dP/dt, were found to be resistant to isoproterenol-induced cardiac hypertrophy, and presented a reduction of ischemia-reperfusion induced arrhythmias. Moreover, TGR(A1-7)3292 presented improved cardiac function after ischemia-reperfusion in isolated hearts and protection against cardiac dysfunction, fibrosis and hypertension when challenged with DOCA-salt (Santiago et al. 2010). In the present study, we extended these observations by showing that acute or chronic blockade of Ang-(1-7) in these animals led to marked changes in the cardiac and vascular autonomic nervous system modulation.

Corroborating previous results (Botelho Santos et al. 2007; Santos et al. 2004), the evaluation of cardiovascular parameters showed that TGR groups at baseline condition did not present neither blood pressure nor autonomic alterations when compared to control rats. A chronic elevation of Ang-(1-7) in this model is induced through genetic manipulation, leading to an overexpression of a fusion protein which produces Ang-(1-7) after hydrolysis by furine. This manipulation also induces a systemic alteration which establishes a new cardiovascular homeostasis condition, characterized by increased cardiac output and decreased peripheral resistance, leading to the absence of gross changes in systemic blood pressure (Botelho Santos et al. 2007).

We observed that the acute blockade of Ang-(1-7) in TGR group produced a dramatic enhancement in blood pressure after 2 hours of A-779 administration (over 70 mmHg in systolic blood pressure), thus suggesting that their normal blood pressure levels are dependent on compensatory mechanisms. This issue awaits further studies to be clarified. On the other hand, the chronic disruption of Ang-(1-7) actions in this model caused by A-779 did not change blood pressure. Whether this could be due to adaptive alterations, such as increased formation of alamandine, remains to be investigated (Lautner et al. 2013). Nevertheless, some of the chronic, and even the acute, effects of Ang-(1-7) in SD rats can only be reverted by D-Pro<sup>7</sup>-Angiotensin-(1-7) which blocks both alamandine and Ang-(1-7) (Herath et al. 2013; Klein et al. 2013; Kluskens et al. 2009; Silva et al. 2007).

The TGR group, which received the Mas receptor antagonist A-779 acutely for 7 days, presented increases in sympathovagal balance, systolic

blood pressure variability, and vascular sympathetic modulation, suggesting that different mechanisms underlie the autonomic modulation and blood pressure control influenced by Ang-(1-7).

In the current view of the RAS, in which different axis affect cardiovascular homeostasis, it is known that central or peripheral Ang II elevations cause sympathetic overactivity and decrease heart rate variability and baroreflex sensitivity (Campagnole-Santos et al. 1988; McKinley et al. 2003), while the opposite effects are exerted by Ang-(1-7) and possibly by alamandine (Santos 2014). As observed in Figure 1, Ang-(1-7) injection in SD rats induces an increase in parasympathetic modulation, starting two hours after treatment. This effect was apparently independent of the reduction in systolic and diastolic pressures 5 hours after the injection. The effect of acute Ang-(1-7) administration, first in cardiac modulation, followed by a later alteration in blood pressure, once more denotes the possible differential role of the peptide on central mechanisms governing these parameters (Feng et al. 2010; Zimmerman et.al 2004;).

In order to evaluate the effects produced by Ang-(1-7)-Mas receptor axis, other studies have used A-779 (Ferreira et al. 2011; Simões & Silva et al. 2013). In the present study, the acute administration of A-779 in control rats did not produce any effect when compared to saline administration. The absence of alterations may indicate that, in basal conditions, circulating Ang-(1-7) and Ang-(1-7)-Mas interaction did not have a role in blood pressure levels, although regional blood flow and cardiac output changes could be masking each other as

to blood pressure values (Sampaio et al. 2003). This seemed to be particularly true in anesthetized rats (Botelho Santos et al. 2007; Sampaio et al. 2003).

In the TGR(A1-7)3292 with increased levels of Ang-(1-7) (Botelho Santos et al. 2007; Santos et al. 2004), A-779 injection induced expressive alterations in cardiac and vascular parameters: heart rate, systolic and diastolic blood pressures were increased two hours after A-779 injection. Additionally, blood pressure variability and the low frequency component (LF) of blood pressure variability were also increased. These effects remained for 2 hours after the injection, denoting the possibility of activation of reverberating signaling pathways, in addition to the acute signaling mechanisms changes, possibly induced by the peptide.

It should be stressed that a direct effect of Ang-(1-7) in cardiac tissue might also contribute to cardiovascular hemostasis, as suggested by some studies demonstrating a cardioprotective effect in TGR(A1-7)3292, while the genetic deletion of Mas receptor induced cardiac dysfunction (Guimarães et al. 2012). More recently, an important modulation of cardiac  $\beta$ -adrenergic activity by Ang-(1-7) was also reported (Martins Lima et al. 2013; Oscar et al. 2015).

In conclusion, the results obtained in this study suggest that there is an close interaction between autonomic nervous system and the Ang-(1-7)-Mas receptor axis. While the stimulation of Ang-(1-7)-Mas receptor axis by Ang-(1-7) induces parasympathetic-predominant cardiovascular effects, its inhibition by A-779 promotes sympathetic-predominant responses. Therefore, the mechanisms of an adequate control of circulation seem to be not only dependent on the autonomic nervous system and the well-known humoral mechanisms, but also

on the Ang-(1-7)-*Mas* receptor axis, which impacts autonomic modulation. This means that, either to the sympathetic or the parasympathetic branch, alterations in the Ang-(1-7)-*Mas* receptor axis may be able to stimulate the capacity of the autonomic nervous system to generate physiological responses. Since there has been a growing interest in the pharmacological manipulation of the Ang-(1-7)-*Mas* receptor axis for blood pressure management, our novel findings may shed some light on the understanding of how Ang-(1-7) can modify cardiovascular homeostasis.

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#### **Disclosure of conflict of interest**

None.

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## Figure Captions

**Figure 1.** Acute effects of intravenous bolus injection of Ang-(1–7) on cardiovascular autonomic control of SD rats (Sprague Dawley - control group). The bars describe temporal assessment from baseline until 5 hours after the injection of saline solution (white bars) and an Ang-(1-7) solution (black bars). The panels illustrate the variations of heart rate variability (HRV) (A), high-frequency (HF) band of HRV in normalized units (nu) (B), systolic arterial pressure (C), and diastolic arterial pressure (D). The results were different when compared to saline injection (\*\* $p < 0.01$  vs. saline). Two-way ANOVA with multiple comparisons followed by Tukey's post hoc test.

**Figure 2.** Acute effects of intravenous bolus injection of A-779 on cardiovascular autonomic control of the TGR group (transgenic rats [TGR(A1-7)3292] overexpressing Ang-(1-7) fusion protein). The bars describe temporal assessment from baseline until 5 hours after the injection of a saline solution (white bars) and A-779 solution (black bars). The panels illustrate systolic arterial pressure (A), diastolic arterial pressure (B), systolic arterial pressure (SAP) variability (C), and the low frequency band of systolic arterial pressure variability (SAP LF) (D). The results were different when compared to saline injection (\*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ). The symbol # indicates differences among TGR groups at different time points: baseline, 1hr and 5 hr (# $p < 0.05$ ; ### $p < 0.01$ ).

Two-way ANOVA with multiple comparisons followed by Tukey's post hoc test.

**Figure 3.** Chronic effects of A-779 on cardiovascular autonomic control. Comparison among SD (Sprague Dawley - control group), TGR (transgenic rats [TGR(A1-7)3292] overexpressing Ang-(1-7) fusion protein), and TGR with osmotic pump containing A-779 for 7 days (TGRop). The panels illustrate the variations of heart rate (A), systolic arterial pressure (B), cardiac sympathovagal balance LF/HF (C), systolic arterial pressure (SAP) variability (D), the low frequency band of systolic arterial pressure variability (SAP LF) (E). The symbols indicate that significant differences were found when compared to the TGR group (\* $p < 0.05$ ; One-way ANOVA followed by Tukey's post hoc test).

**Table 1.** Hemodynamic parameters and cardiovascular autonomic modulation in baseline condition of control (SD) and transgenic rats (TGR)

	SD	TGR
<b>SAP (mmHg)</b>	111.92 ± 7.37	112.88 ± 10.74
<b>DAP (mmHg)</b>	61.41 ± 5.81	63.65 ± 3.41
<b>HR (bpm)</b>	318.99 ± 35.47	335.94 ± 30.71
<b>HRV-var (s<sup>2</sup>)</b>	22.10 ± 6.06	18.22 ± 10.10
<b>LF (nu)</b>	12.83 ± 7.40	14.92 ± 15.63
<b>HF (nu)</b>	87.10 ± 7.44	84.56 ± 16.77
<b>LF/HF</b>	0.18 ± 0.10	0.23 ± 0.30
<b>SAPV</b>	8.31 ± 5.35	6.44 ± 2.04
<b>VLF</b>	4.03 ± 3.84	4.19 ± 4.23
<b>LF</b>	2.89 ± 2.48	1.83 ± 1.66
<b>HF</b>	1.10 ± 0.90	1.72 ± 1.43
<b>α-index (ms/mmHg)</b>	0.99 ± 0.52	1.70 ± 2.61

SAP = systolic arterial pressure, DAP=diastolic arterial pressure, HR = heart rate, HRV-var = total variance of heart rate variability, LF = low frequency, HF = high frequency, VLF = very low frequency, LF/HF = sympathovagal balance, SAPV = systolic arterial pressure variability, α-index = spontaneous baroreflex sensitivity.

Figure 1

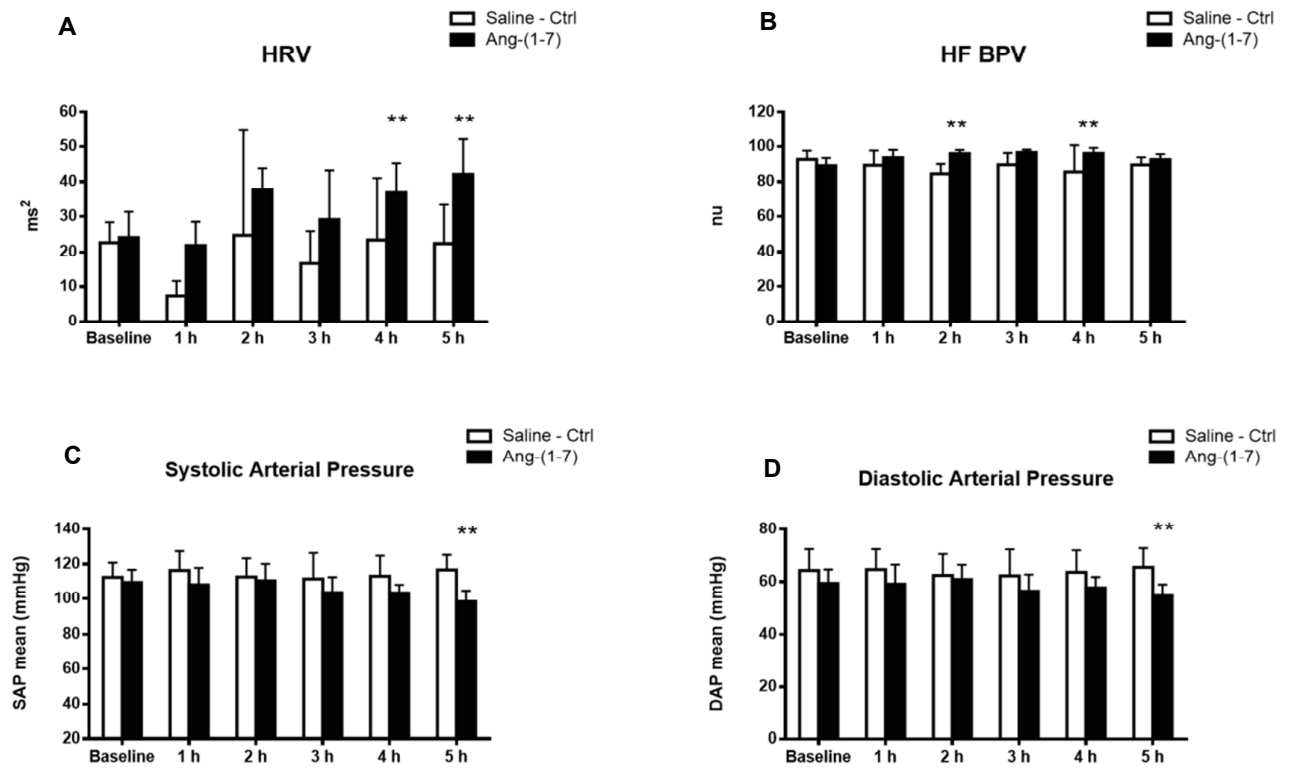
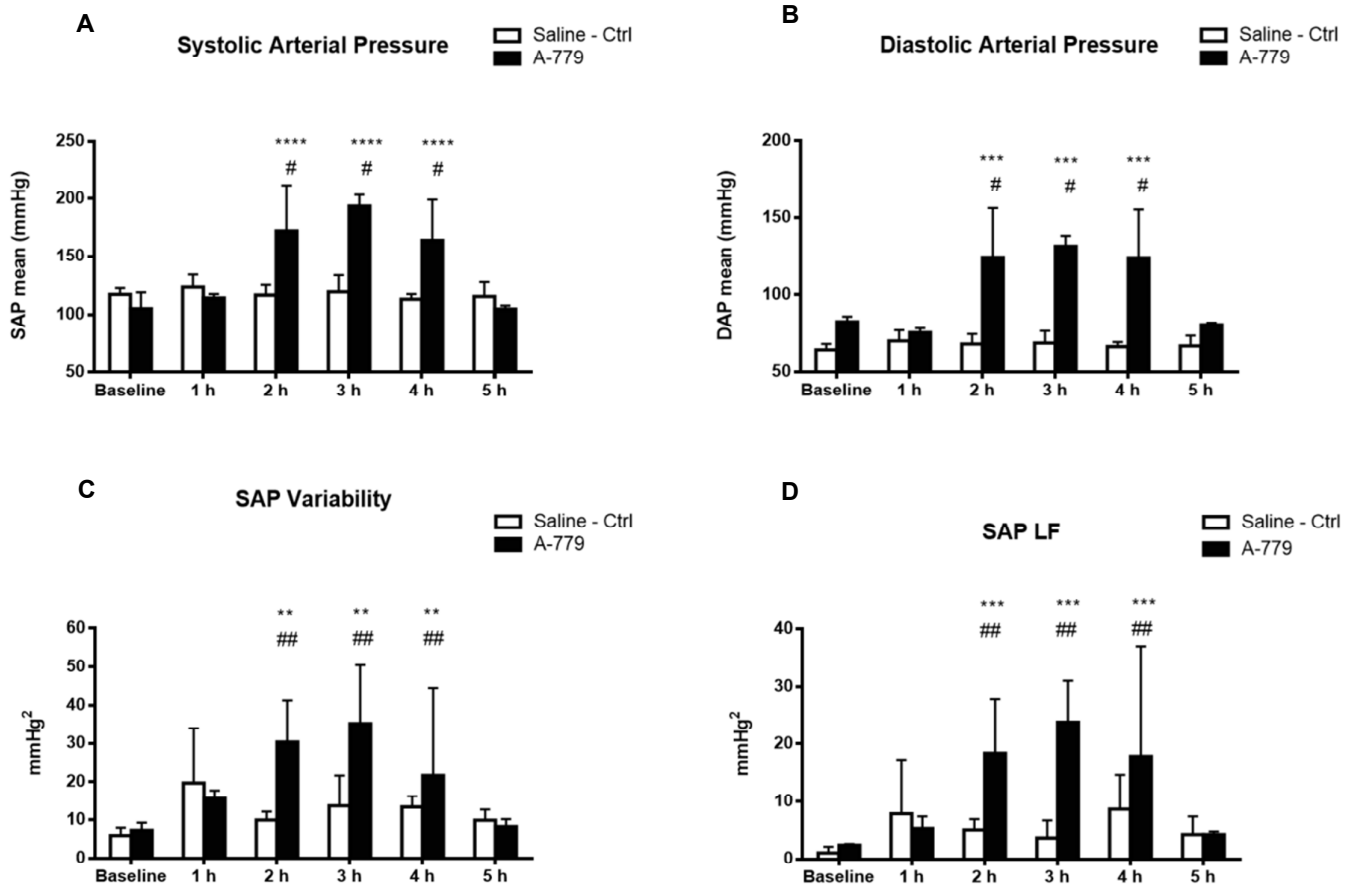
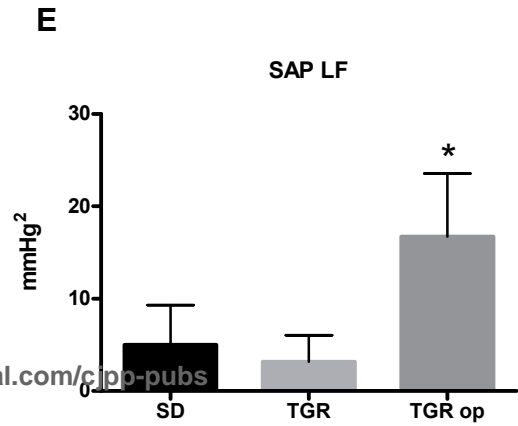
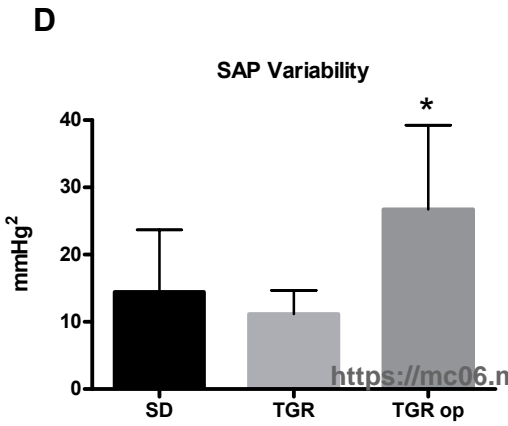
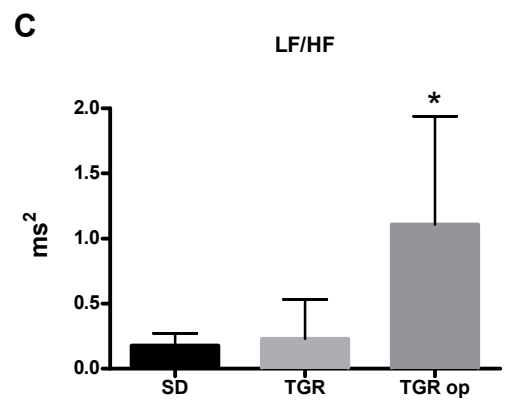
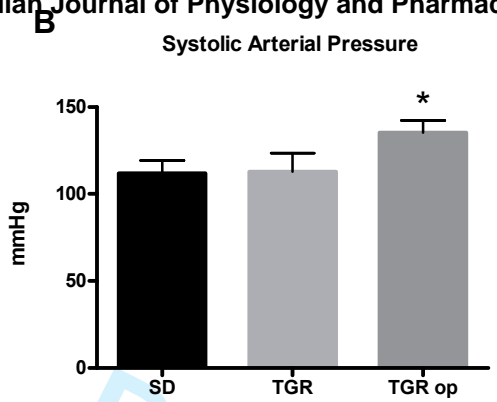
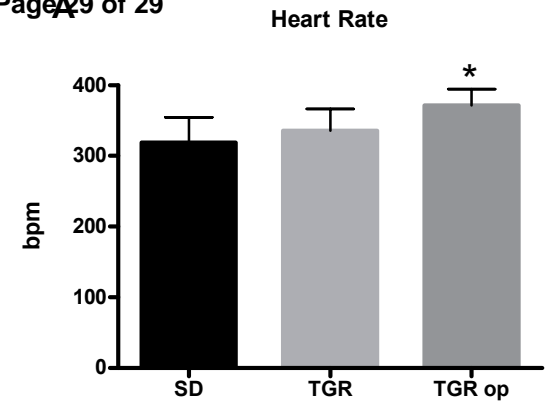


Figure 2





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