Metabolic Syndrome

Increasing Angiotensin-(1–7) Levels in the Brain Attenuates Metabolic Syndrome–Related Risks in Fructose-Fed Rats

Priscila S. Guimaraes, Mariana F. Oliveira, Janaína F. Braga, Ana Paula Nadu, Ann Schreihofer, Robson A.S. Santos, Maria Jose Campagnole-Santos

Abstract—We evaluated effects of chronic intracerebroventricular infusion of angiotensin (Ang)-(1–7) on cardiovascular and metabolic parameters in fructose-fed (FF) rats. After 6 weeks of fructose intake (10% in drinking water), Sprague-Dawley rats were subjected to intracerebroventricular infusion of Ang-(1–7) (200 ng/h; FF+A7 group) or 0.9% sterile saline (FF group) for 4 weeks with continued access to fructose. Compared with control rats, FF rats had increased mean arterial pressure and cardiac sympathetic tone with impaired baroreflex sensitivity. FF rats also presented increased circulating triglycerides, leptin, insulin, and glucose with impaired glucose tolerance. Furthermore, relative weights of liver and retroperitoneal adipose tissue were increased in FF rats. Glycogen content was reduced in liver, but increased in muscle. In contrast, fructose-fed rats subjected to chronic intracerebroventricular infusion of Ang-(1–7) presented reduced cardiac sympathetic tone with normalized mean arterial pressure, baroreflex sensitivity, glucose and insulin levels, and improved glucose tolerance. Relative weight of liver, and hepatic and muscle glycogen contents were also normalized in FF+A7 rats. In addition, FF+A7 rats had reduced mRNA expression for neuronal nitric oxide synthase and NR1 subunit of N-methyl-d-aspartate receptor in hypothalamus and dorsomedial medulla. Ang-(1–7) infusion did not alter fructose-induced hyperleptinemia and increased relative weight of retroperitoneal adipose tissue. There were no differences in body weights, neither in liver mRNA expression of phosphoenolpyruvate carboxykinase or glucose-6-phosphatase among the groups. These data indicate that chronic increase in Ang-(1–7) levels in the brain may have a beneficial role in fructose-fed rats by ameliorating cardiovascular and metabolic disorders. (Hypertension. 2014;63:1078-1085.) • Online Data Supplement

Key Words: autonomic nervous system ■ insulin resistance ■ metabolic syndrome

Metabolic syndrome (MetS) is currently recognized as a worldwide health problem,1,2 and the substantial increase in fructose intake in human diet, especially as high-fructose corn syrup, has been considered a potential factor predisposing humans to MetS.3,4 MetS is a cluster of different risk factors, such as increase in visceral fat deposition, glucose intolerance, dyslipidemia, and hypertension, which increases the risk of development of cardiovascular and renal diseases and type II diabetes mellitus.2,5

Recent studies in rodents have shown that the renin–angiotensin system, through the inappropriate overactivity of angiotensin (Ang) II on Ang II type 1 (AT₁) receptor especially in the circulation and in the white adipose tissue, plays a pivotal role on the pathogenesis of MetS.6–8 Peripheral blockade of Ang II synthesis or its action on AT₁ receptor attenuates metabolic disorders in obese rodents.6–8 In contrast, the other functional arm of renin–angiotensin system, represented by the actions of Ang-(1–7) on Mas receptor, has been recognized widely as a counter-regulatory axis of Ang II/AT₁ receptor actions.

Recent studies have shown that chronic increases in peripheral Ang-(1–7)/Mas receptor activity play a protective role in the regulation of cardiovascular and energy metabolism. Transgenic rats with increased Ang-(1–7) levels in the circulation [TGR(A1–7)3292] are protected against cardiac dysfunction, fibrosis, and hypertension when subjected to deoxycorticosterone (DOCA) acetate-salt model9 and have enhanced resting glucose and lipid metabolism.10 Accordingly, Giani et al.11,12 showed that chronic subcutaneous infusion of Ang-(1–7) improved insulin resistance, hypertension, and cardiac remodeling in fructose-fed rats. However, genetic deletion of Mas receptor alters glucose and lipid metabolism and the neural control of blood pressure, inducing a state of MetS.13–15

Taken together, these data provide a clear evidence of a protective role of peripheral Ang-(1–7)/Mas receptor axis in the regulation of cardiovascular system and energy metabolism. Our group showed recently that chronic increase in Ang-(1–7) levels in the brain attenuates DOCA-salt hypertension, and that this effect is related to a dramatic beneficial role.
of Ang-(1–7) on autonomic activity to the heart. Emerging evidence has also suggested that the sympathoinhibitor neurotransmitter nitric oxide (NO)\textsuperscript{17–19} and the sympathoexcitatory neurotransmitter glutamate, through \textit{N}-methyl-\textit{d}-aspartate receptor (NMDAr),\textsuperscript{20–23} play a role on energy metabolism. Recent data have shown that central lipid infusion by the carotid artery deregulates hepatic insulin signaling in rats, in part, by increasing neuronal NO synthase (nNOS) activity in hypothalamus, suggesting that nNOS in the hypothalamus might be involved with the development of insulin resistance.\textsuperscript{19} Moreover, blockade of NMDAr in the perifornical region of lateral hypothalamus prevents orexigenic effects of neuropeptide Y.\textsuperscript{21}

In the present study, we hypothesized that chronic increase in Ang-(1–7) levels in the brain could improve cardiovascular and metabolic disorders in rats with MetS. To address this hypothesis, fructose-fed rats were subjected to chronic intracerebroventricular infusion of Ang-(1–7) and were evaluated for cardiovascular and metabolic parameters. In addition, mRNA levels of Mas receptor, nNOS, and NR1 subunit of NMDAr (NR1/NMDAr) in brain areas were also evaluated.

Methods
An expanded Methods section detailing the techniques and procedures performed is provided in the online-only Data Supplement.

Animals
Sprague-Dawley rats, 6 to 7 weeks old at the beginning of the experiment, from the animal facilities of the Laboratory of Hypertension, Federal University of Minas Gerais (UFMG), Brazil, were used. Protocols were approved by the institutional committee that regulates the use of laboratory animals (Comitê de Ética em Experimentação Animal–CETEA/UFMG, protocol 195/2011) and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Rats, randomly housed in groups of 2 to 4 rats per cage in a controlled environment (12:12-hour light–dark cycle; lights on 6:00 am; room temperature, 22°C–24°C), were fed standard chow ad libitum. The control group received tap water to drink and the others received 10% fructose solution to drink, prepared every 2 days in tap water.

Chronic Intracerebroventricular Infusion
After 6 weeks of fructose intake, rats were subjected to intracerebroventricular infusion (0.25 μL/h for 28 days) of 0.9% sterile saline (FF) or Ang-(1–7) (200 ng/h; FF+A7; Millipore) using an osmotic minipump (ALZET, Model 2004). The access to fructose continued during the 4 weeks of intracerebroventricular infusion. Control-fed rats were also subjected to intracerebroventricular infusion of 0.9% sterile saline (CTL) or Ang-(1–7) (CTL+A7).

Serum and Tissue Measurements
Serum metabolic parameters were measured using enzymatic or ELISA assays. The homeostasis model assessment of insulin resistance (HOMA-IR) was obtained.\textsuperscript{24} The glycogen content of liver and of the enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) in the liver were determined by quantitative reverse transcriptase PCR. Gene expression was normalized to the endogenous ribosomal S26 and analyzed after Livak and Schmittgen method.\textsuperscript{25}

Real-Time Polymerase Chain Reaction
The mRNA expression of Mas receptor, nNOS, and NR1/NMDAr in the brain and of the enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) in the liver were determined by quantitative reverse transcriptase polymerase chain reaction (PCR) using SYBR Green reagent (Applied Biosystems) in ABI 7900 or ViiA7 platform (Applied Biosystems). Gene expression was normalized to the endogenous ribosomal S26 and analyzed after Livak and Schmittgen method.\textsuperscript{25}

Protocol 1: Effects of Fructose Feeding and Intracerebroventricular Ang-(1–7) on Autonomic Regulation of Heart Rate in Conscious Freely Moving Rats
At the end of the 10th week, anesthetized (mixture of ketamine [80 mg/kg] and xylazine [6 mg/kg]) rats were subjected to femoral artery and vein catheters to record arterial pressure (AP) and inject drugs, respectively. After 1 day of recovery, AP, mean AP (MAP, in mm Hg), and heart rate (HR; bpm) were monitored continuously by a data acquisition system (Accqknowledge software 4.1, Biopac System, CA). The baroreflex bradycardia (in ms/mm Hg)\textsuperscript{16,26} and the cardiac autonomic tone (ΔHR, in bpm)\textsuperscript{16} were evaluated.

Protocol 2: Effects of Fructose Feeding and Intracerebroventricular Ang-(1–7) on Metabolic Parameters, Body and Tissue Weights, mRNA Expression of PEPCK, G6Pase, nNOS, Mas Receptor, and NR1/NMDAr
Rats were weighed at the onset of the access to fructose (or tap water) and every week during the 10-week protocol. On the 10th week, rats were fasted overnight and subjected to glucose tolerance test the next morning. At =9:00 am, blood samples (drop from tail with 26G needle) from conscious fasted rats were taken immediately before the injection of glucose (2 g/kg; 0.5 g/mL, IP) and 15, 30, 60, and 120 minutes after injection. Blood glucose levels were measured using a glucometer (Optium Xceed, Abbott). After 3 days, rats were fasted for 6 to 8 hours (starting at 6:00 am) and then decapitated. The trunk blood was collected and fresh body tissues were weighed and quickly frozen in dry ice for subsequent analysis. The weights of the left ventricle, epididymal and retroperitoneal adipose tissues, and liver were normalized to final body weight (BW). The brain was removed for dissection of the hypothalamus and dorsomedial medulla. The dissected brain tissue and liver fragment were immediately frozen in dry ice and kept at −80°C for later analysis of mRNA expression by quantitative reverse transcriptase PCR.

Statistical Analysis
Data are expressed as mean±SEM. Differences among groups were analyzed by 2-way ANOVA repeated measures for the glucose tolerance test and by 1-way ANOVA for all other measures. Significant ANOVA analyses were followed by Student–Newman–Keuls post hoc tests. The criterion for statistical significance was set at \(P<0.05\).

Results
Cardiovascular Parameters
As shown in Figure 1, FF rats presented increased baseline MAP (118±2 mm Hg), impaired baroreflex control of HR (0.53±0.05 ms/mm Hg), and increased cardiac sympathetic tone (112±7 bpm) compared with CTL (107±3 mm Hg; 0.96±0.08 ms/mm Hg; 94±3 bpm, respectively). In contrast, FF+A7 rats had normalized MAP (108±3 mm Hg) and the baroreflex bradycardia sensitivity index (0.96±0.07 ms/mm Hg; Figure 1). In addition, cardiac sympathetic tone in FF+A7 rats was reduced compared with both CTL and FF rats (77±4 bpm). Chronic intracerebroventricular infusion of Ang-(1–7) did not alter cardiovascular parameters in control-fed rats (Table S2 in the online-only Data Supplement). There was no difference in baseline HR, cardiac parasympathetic tone, or intrinsic HR among all groups (Figure 1).

BW Gain and Relative Tissue Weight
Data for body and relative tissue weights from CTL, FF, and FF+A7 rats are summarized in Table S3. BW gains through the 10-week protocol and final BWs were comparable in CTL,
FF, and FF+A7 rats. Relative weight of retroperitoneal adipose tissue was increased in both FF and FF+A7 rats compared with CTL. Differently, although FF rats had increased relative weight of liver, FF+A7 rats presented lower relative weight of liver, comparable with CTL. There was no difference in relative weight of epididymal adipose tissue or left ventricle among all groups.

**Serum Analysis**

As shown in Figure 2, FF rats had elevated serum glucose (136±4 mg/dL) and insulin (1.8±0.1 ng/mL), as well as HOMA-IR index (18.1±1) compared with CTL (121±3 mg/dL; 1.3±0.1 ng/mL; 11.4±2, respectively). In contrast, FF+A7 rats had serum glucose (125±4 mg/dL) and insulin (1.4±0.1 ng/mL), and HOMA-IR index score (12.7±2) restored to levels seen in CTL rats. Serum levels of leptin (4.0±0.1 ng/mL) and triglycerides (222±13 mg/dL) were increased in FF rats compared with CTL (3.0±0.3 ng/mL and 151±10 mg/dL, respectively) and remained elevated in FF+A7 rats (4.2±0.3 ng/mL and 229±12 mg/dL, respectively) in parallel with the excess retroperitoneal adipose tissue. The FF+A7 rats displayed slightly increased levels of high-density lipoprotein (HDL; 22±1 mg/dL) compared with CTL (19±1 mg/dL) and FF rats (18±1 mg/dL; Figure 2), but total cholesterol was not different among the groups (58±2 mg/dL in 16 CTL rats, 55±2 mg/dL in 12 FF rats, and 60±3 mg/dL in 11 FF+A7 rats). Chronic intracerebroventricular infusion of Ang-(1–7) did not alter serum metabolic parameters in control-fed rats (Table S4).

**Glucose Tolerance**

Injection of d-glucose increased serum glucose levels in all rats. However, blood glucose rise was significantly higher in FF rats compared with CTL at 15, 30, and 60 minutes after the injection (Figure 3A), resulting in a larger area under the curve for d-glucose–induced changes in blood glucose (Figure 3B). Remarkably, chronic intracerebroventricular
infusion of Ang-(1–7) normalized the response to d-glucose in FF+A7 rats at all time points measured (Figure 3A), which resulted in similar area under the curve to CTL (Figure 3B). The response to d-glucose injection was comparable between CTL+A7 and CTL rats (Figure S1).

Hepatic and Muscle Glycogen Content
In comparison with CTL (17.4±1.0 mg/g), hepatic glycogen was reduced by 60% in FF rats (10.4±2.2 mg/g; Figure 4A), and muscle glycogen was elevated by 150% (0.61±0.08 versus 0.22±0.05 mg/g in CTL; Figure 4B). In contrast, hepatic (18.9±2.6 mg/g) and muscle (0.31±0.07 mg/g) glycogen levels were normalized in FF+A7 rats, being comparable with CTL (Figure 4).

mRNA Expression of PEPCK and G6Pase in the Liver
The mRNA expression of PEPCK or G6Pase was comparable among the groups (Table S5).

mRNA Expression of Mas Receptor, nNOS, and NR1/NMDAr in the Hypothalamus and Dorsomedial Medulla
As shown in the Table, the mRNA expression of nNOS and NR1/NMDAr in the hypothalamus and dorsomedial medulla was comparable between CTL and FF rats. Differently, intracerebroventricular infusion of Ang-(1–7) in fructose-fed rats significantly reduced mRNA expression of nNOS and NR1/NMDAr in these brain regions. The mRNA expression of Mas receptor in the hypothalamus was comparable among the groups.

Discussion
The present study addressed cardiovascular and metabolic effects of chronic increase in Ang-(1–7) levels in the brain in fructose-fed rats. The major findings were that fructose-fed rats receiving Ang-(1–7) intracerebroventricular infusion had normalized baseline MAP, baroreflex control of HR, and reduced cardiac sympathetic tone. Interestingly, along with these cardiovascular improvements, these rats presented normalized glucose tolerance, glycemia, insulinemia, and HOMA score. Moreover, FF+A7 rats had increased HDL and normalized hepatic and muscle glycogen content. Molecular analysis of brain areas revealed that FF+A7 rats had reduced mRNA expression of nNOS and NR1/NMDAr in the hypothalamus and in the dorsomedial medulla. These data suggest that increasing Ang-(1–7) levels in the brain may play a beneficial role against cardiovascular and metabolic disorders induced by chronic fructose intake.

In line with previous studies performed in mice,27,28 rats,29,30 or humans,3 FF rats presented slightly increased baseline MAP, impaired baroreflex control of HR, and increased cardiac sympathetic tone, reinforcing the deleterious effects of high fructose intake on cardiovascular system. Interestingly, chronic
increase in Ang-(1–7) levels in the brain normalized MAP, the baroreflex control of HR, and reduced cardiac sympathetic activity in FF+A7 rats, as previously observed in DOCA-salt hypertensive rats. Intriguingly, cardiac sympathetic tone in FF+A7 rats was slightly lower than CTL, which might be because of the duration of intracerebroventricular infusion, which was longer than the previous study. In addition, questioning whether chronic infusion of Ang-(1–7) in the brain would reduce sympathetic activity or baseline MAP in normotensive conditions could be raised. In control rats (CTL+A7), 28-day intracerebroventricular infusion of Ang-(1–7) did not alter baseline MAP and the baroreflex control of HR. However, we have observed previously that 21-day infusion improved the baroreflex bradycardia without changing baseline MAP in lean Zucker rats. Future studies will be necessary to address whether such difference might be related to the duration of infusion, strain, or other aspects. Regardless, the present data indicate that chronic increase in brain Ang-(1–7) levels may induce beneficial cardiovascular effects in pathophysiological conditions, such as fructose-induced MetS or DOCA-salt model.

Along with the cardiovascular changes, FF rats presented noticeable glucose intolerance. Fructose is considered a lipogenic sugar, leading to increased triglycerides deposition in white adipose tissues, hypertriglyceridemia, proinflammatory state, and increased ectopic fat deposition, especially in the liver and skeletal muscle. The increased glycemic response curve in FF rats compared with CTL during the glucose tolerance test, along with the increased levels of serum insulinemia and glycemia and increased HOMA-IR index, as well as the reduced hepatic glycogen content, suggest that FF rats present reduced response of insulin-sensitive tissues, a state of insulin resistance. In contrast, FF+A7 rats had all those parameters normalized, suggesting a dramatic beneficial effect of increasing Ang-(1–7) levels in the brain in preventing the development of insulin resistance.

Different studies have shown that changes in autonomic activity in favor of the sympathetic branch to different organs have been identified in animals with MetS. Sympathectomy or pharmacological blockade of sympathetic activity prevents the development of insulin resistance, hyperinsulinemia, and hypertension in fructose-fed rats, suggesting that these complications might depend on sympathetic overactivity. However, insulin resistance has been observed with hepatic parasympathectomy or pharmacological blockade. Reduced hepatic parasympathetic activity has already been suggested as one of the mechanisms underlying insulin resistance in sucrose-fed rats. A limitation of the present study was that we were not able to evaluate autonomic balance to other targets, such as the liver and skeletal muscle. Future studies will be necessary to address the mechanisms underlying Ang-(1–7) effects on glucose metabolism.

Equally observed in the liver, one would expect that muscle glycogen content would be reduced in FF rats. However, we observed the opposite. Chronic activation of the AMP-activated protein kinase pathway in the liver, which stimulates glucose uptake independently of insulin signaling, could be a possible mechanism accounting for the increased muscle glycogen content in FF rats. Interestingly, FF+A7 rats presented muscle glycogen content similar to CTL rats. Studies have shown that stimulation of ventromedial hypothalamus promotes glucose uptake by skeletal muscle, independently of insulinemia.
and that glucose uptake through AMP-activated protein kinase pathway is abolished by sympathetic blockade. Therefore, a reduction of sympathetic activity to other targets, such as the skeletal muscle and liver, could be a possible mechanism underlying the metabolic benefits of increasing Ang-(1–7) in the brain of fructose-fed rats.

Despite no difference was observed for total serum cholesterol and HDL between FF and CTL rats, increasing Ang-(1–7) levels in the brain increased HDL levels in fructose-fed rats, without changing total cholesterol in these rats. Recent study has shown that chronic inhibition of the melanocortin pathway in the brain by pharmacological, genetic, or endocrine mechanisms increases HDL levels in rats, in part, by reducing its uptake by the liver. In addition, this mechanism is related to a vagal-mediated modulation of hepatic pathways controlling cholesterol synthesis and reuptake. Therefore, despite there is no evidence of Ang-(1–7) effects on hepatic parasympathetic activity, we do not rule out the potential of that to be a mechanism underlying the increased levels of HDL, as well as the increased hepatic glycogen content. Another possibility could be a modulation of the melanocortin pathway in the brain by Ang-(1–7).

Ang-(1–7) intracerebroventricular infusion reduced mRNA expression of NR1/NMDAr and nNOS in the hypothalamus of FF rats. It is possible that the alteration in NMDA mRNA expression in the hypothalamus might be related to the reduced sympathetic activity in FF+A7, and as a consequence, the reduced cardiac sympathetic tone, the improvement on baroreflex control of HR, and the improvement on glucose metabolism observed in these rats. Increased NMDA activity has been related to the higher sympathoexcitation during hypertension or heart failure. Another possibility would be the reduction of the activity of hypothalamic neurons directly related to the energy balance. However, the reduction on mRNA expression of nNOS in the hypothalamus could be related to NO effect in hypothalamic neurons related to the modulation of with hepatic sensitivity to insulin. The mRNA expression of nNOS was also reduced in the dorsomedial medulla of FF+A7 rats, whereas there was no change in FF rats. It has been suggested that, acutely, Ang-(1–7) increases NO synthesis in the brain. However, our data showed that chronically Ang-(1–7) reduces mRNA expression of nNOS in both hypothalamus and dorsomedial medulla. The functional significance of increasing Ang-(1–7) in the brain chronically on genetic transcription of nNOS in these areas is still to be elucidated. Differently, there was no difference in mRNA expression of Mas receptor among the groups. The modulation of mRNA expression of Mas receptor by Ang-(1–7) is not fully elucidated. It has been shown that increasing circulating or tectidual Ang-(1–7) levels can either not alter, increase, or reduce mRNA expression of Mas receptor in different tissues.

Considering that both nNOS and NMDAr are expressed by different types of neurons and are involved with different physiological responses, and that we evaluated the whole regions, interpretation of mRNA data becomes somehow limited. Changes in the protein expression and activity, as well as in the activity of other intracellular pathways, because of the fructose intake or Ang-(1–7) infusion are still to be addressed. Besides that, the present study suggests that chronic increase in Ang-(1–7) levels in the brain in fructose-fed rats might be, directly or indirectly, related to a reduction in gene transcription of nNOS enzyme and NR1/NMDAr in different brain regions.

**Perspectives**

The Ang-(1–7)/Mas receptor is a renin–angiotensin system axis majorly known for its opposing effects to Ang II/AT1 receptor in different pathological conditions. In the brain, Ang-(1–7) is primarily known for its beneficial effects on the regulation of cardiovascular system. The present study extends the previous ones by showing that a chronic increase in Ang-(1–7) levels in the brain improves both cardiovascular and metabolic parameters in an experimental model of MetS. Chronic intracerebroventricular infusion of Ang-(1–7) attenuated the development of hypertension and improved the baroreflex control of HR and the glucose metabolism in FF rats. This study also supports pursuit of pharmacological strategies that promote increased Ang-(1–7) levels in the brain to ameliorate the metabolic and cardiovascular disorders in the setting of MetS.

**Acknowledgments**

We are thankful to Marilene Luzia Oliveira, José Roberto da Silva, Bônia Aparecida Alves da Cruz, and Mônica Alves da Cruz for technical assistance.

**Sources of Funding**

This study was funded by Conselho Nacional de Desenvolvimento Científico e Tecnológico and Fundação de Amparo a Pesquisa do Estado de Minas Gerais through INCT-Nanobiofar and Programa de Núcleos de Excelência (PRONEX-#CBB-APQ-04758-10), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

**Table. Effect of Chronic Intracerebroventricular Infusion of Ang-(1–7) on mRNA Expression of Mas Receptor, nNOS, and NR1/NMDAr in Autonomic Regulatory Regions of the Brain in Fructose-Fed Rats**

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Hypothalamus</th>
<th>Dorsomedial Medulla</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mas Receptor</td>
<td>nNOS</td>
</tr>
<tr>
<td>CTL (n=4–5)</td>
<td>1.00 (1.251–0.799)</td>
<td>1.00 (1.085–0.921)</td>
</tr>
<tr>
<td>FF (n=4–7)</td>
<td>1.05 (1.131–0.984)</td>
<td>0.99 (1.030–0.947)</td>
</tr>
<tr>
<td>FF+A7 (n=5–7)</td>
<td>1.56 (1.689–1.442)</td>
<td>0.76 (0.820–0.711)†</td>
</tr>
</tbody>
</table>

Values are fold changes in mRNA expression in relation to the control group. Group sizes are as n. Ang-(1–7) indicates angiotensin-(1–7); CTL, control; FF, fructose-fed with intracerebroventricular infusion of 0.9% sterile saline; FF+A7, fructose-fed with intracerebroventricular infusion of Ang-(1–7) rats; nNOS, neuronal nitric oxide synthase; and NR1/NMDAr, NR1 subunit of the N-methyl-D-aspartate receptor.

*P<0.05 vs CTL rats.
†P<0.05 vs FF rats. Data were analyzed by 1-way ANOVA followed by Newman–Keuls post hoc test.
Disclosures
None.

References

### Novelty and Significance

**What Is New?**

Along with the beneficial cardiovascular effects, chronic increase in angiotensin-(1–7) in the brain in fructose-fed rats:
- prevented insulin resistance,
- normalized hepatic and muscle glycogen contents, and
- increased high-density lipoprotein levels.

**What Is Relevant?**

Chronic increase in angiotensin-(1–7) in the brain ameliorates risk factors for cardiovascular diseases in fructose-fed rats:
- normalized blood pressure and baroreflex control,
- reduced sympathetic activity, and
- prevented hyperinsulinemia and insulin resistance.

**Summary**

Our data provide a clear evidence of a protective role of angiotensin-(1–7) in the brain for the regulation of cardiovascular system and glucose metabolism in fructose-induced metabolic syndrome.
Increasing Angiotensin-(1–7) Levels in the Brain Attenuates Metabolic Syndrome–Related Risks in Fructose-Fed Rats
Priscila S. Guimaraes, Mariana F. Oliveira, Janaína F. Braga, Ana Paula Nadu, Ann Schreihofer, Robson A.S. Santos and Maria Jose Campagnole-Santos

Hypertension. 2014;63:1078-1085; originally published online February 10, 2014; doi: 10.1161/HYPERTENSIONAHA.113.01847
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/63/5/1078

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2014/02/10/HYPERTENSIONAHA.113.01847.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
Increasing angiotensin-(1-7) levels in the brain attenuates metabolic syndrome-related risks in fructose-fed rats

Priscila S Guimaraes¹, Mariana F Oliveira¹, Janaína F Braga¹, Ana Paula Nadu¹, Ann Schreihofer² Robson AS Santos¹, Maria Jose Campagnole-Santos¹.

¹National Institute of Science and Technology-Nanobiofar, Department of Physiology and Biophysics, Biological Science Institute, Federal University of Minas Gerais, Brazil
²Department of Integrative Physiology, University of North Texas Health Science Center, USA

Running head: Cardiometabolic actions of brain Ang-(1-7)

Corresponding author: Maria Jose Campagnole-Santos
Depto de Fisiologia e Biofisica
Universidade Federal de Minas Gerais
Av. Antonio Carlos, 6627 – ICB
31270-901; Belo Horizonte, MG
55-31-3409-2951; 55-31-3409-2956
Email: mjcs@icb.ufmg.br
METHODS (SUPPLEMENT)

General procedures
A single dose of a broad-spectrum veterinary antibacterial agent (Veterinary Pentabiotic, 2,400 UI/ 0.1 ml, i.m., Ford Dodge), and an analgesic and antiinflammatory agent (Flunixin Meglumine, 1 mg/ kg, s.c., Schering-Plough) were given to rats immediately after the ICV and catheters implantation surgeries.

Chronic ICV infusion
After 6 weeks of fructose intake, rats were anesthetized with Ketamine (80 mg/ kg; Dopalen 10% Vetbrands) and Xylazine (6 mg/kg; Dopase 2% Hertape Calier) and subjected to surgery for implantation of ICV cannula. After removal of the overlying skull bone, the tip of the cannula was placed into the right lateral ventricle (1.5 mm caudal to bregma, 1.5 mm lateral to the midline, 4.5 mm ventral to the dorsal surface of the brain) and fixed to the skull. The cannula was connected via vinyl tubing to an osmotic minipump (ALZET, Model 2004) that was implanted subcutaneously between the scapulae.1 The ventricular infusion site was verified postmortem after injection of Alcian blue dye (2%/ 5 µl) through the ICV cannula (Protocol 1) or by the track of the cannula to the lateral ventricle (Protocol 2).

Evaluation of the Baroreflex Control of Heart rate (HR)
The baroreflex control of HR was evaluated by measuring the reflex changes in HR (converted to pulse interval, PI) in response to transient increases in MAP (mmHg) induced by bolus injections of phenylephrine (PE; 2.5 - 50 µg/ml in 0.1 ml, i.v., Sigma), as described previously.2,3 The baroreflex sensitivity index of each rat was calculated by the average of the ratios (ΔPI/ΔMAP) of maximal changes with each dose of PE. For illustrative purposes only, the best fit line that correlates mean changes in PI and mean changes in MAP for the entire group was plotted, as well.

Cardiac autonomic tone measurement
Autonomic tone to the heart was evaluated in two consecutive days starting 30-60 min after baroreflex analysis. On the first day, rats were subjected to methyliatropine, a muscarinic blocker (3 mg/kg, i.v., Sigma) and the maximum HR was obtained; 15 minutes later, propranolol - a β-adrenergic blocker (4 mg/kg, i.v., Sigma) was injected to assess intrinsic HR. On the next day (24 h later), blockers were injected in the reverse order, therefore the minimal HR was measured after propranolol. The intrinsic HR was obtained 15 min after the injection of both blockers at the end of the experiment in each day. For the analysis intrinsic HR was considered the average of the 2 days. The sympathetic tone was calculated by the difference between the maximum HR (first day; ΔHR in beats/ min) and intrinsic HR, and the parasympathetic tone, by the difference between intrinsic HR and the minimum HR (second day; ΔHR in beats/ min).2

Serum and tissue measures
Samples of trunk blood were centrifuged (950 rcf / 20 min at 4˚C) and serum was frozen until later use. Colorimetric kits were used to measure serum levels of glucose (Labtest
Diagnóstica SA, 84-1/500), triglycerides (Doles, Triglycerides liquiform 2/100), total cholesterol (Labtest Diagnóstica SA 76-2/100), and high density lipoprotein fraction of cholesterol (HDL; Bioclin K-071). Serum insulin and leptin were measured by ELISA (Millipore; Rat/ Mouse Insulin EZRMI-13K and Rat Leptin EZRL-83K). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as the product of fasting glucose (in mmol/ l) and insulin (in μU/ ml) divided by 22.5.⁴

**Hepatic and muscle glycogen content**

Hepatic (~1.0 g tissue) and muscle glycogen (~0.5 g tissue) were extracted and determined as glucose following acid hydrolysis. For glycogen extraction, samples were placed in tubes with 30% KOH saturated with Na₂SO₄. Tubes were placed in a boiling water bath for ~1 hour to obtain a homogeneous solution. Absolute ethanol was added to the solution obtained from the alkaline digest. Samples were centrifuged (870 rcf/ 20 minutes, room temperature), the supernatant was carefully discharged, and the glycogen was dissolved in deionized water (2 ml), precipitated with ethanol (4.5 ml) again, and centrifuged. Glycogen was dissolved in deionized water (to complete 10 ml solution) for colorimetric assay with Anthrone (Sigma) 0.2% in H₂SO₄. Anthrone solution was carefully added to glycogen aliquots of the final dilution in the ice bath. Total volume solution was 1 ml. Tubes were placed in a boiling water bath for 10 minutes, and immediately cooled down in ice bath. A standard curve of glucose solution (5 different concentrations) was used for colorimetric assay. Absorbance of samples and standard curve were measured at 620 nm using a spectrophotometer. Glycogen content was calculated and expressed as mg/ g tissue.

**Real-time polymerase chain reaction (qRT-PCR)**

Total RNA from samples of hypothalamus, dorsomedial medulla, and liver were extracted using TRIzol reagent (Invitrogen Corp, San Diego, CA, USA). Extracted total RNA (1 μg) was treated with DNAase and reverse transcribed with Moloney murine leukemia virus (Invitrogen) using random hexamer primers (Table S1). The mRNA expressions of target genes were determined using SYBR Green reagent (Applied Biosystems) in ABI 7900 or ViiA7 platform (Applied Biosystems). Gene expression was normalized to the endogenous ribosomal S26 and analyzed following Livak and Schmittgen method.⁵
REFERENCES


Table S1: Primers sequences used to perform Real-time PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence</th>
</tr>
</thead>
</table>
| NR1/NMDAr | 5’ ATAGTAGCAATCCACCAAGAGCC 3’  
Rv 5’ GTAGCTCGCCCCATCATTCCGGT 3’ |
| nNOS | Fw 5’ GCCATCCAGCGCATAATAGCCAG 3’  
Rv 5’ GAGGGTAGCTCCAAAGATGTGCTC 3’ |
| Mas | Fw 5’ TAGCCATTAGACAGATGTGCA 3’  
Rv 5’ TGTAGTTTAGGCGGTGCTGTC 3’ |
| PEPCK | Fw 5’ TGCCCATGCAAGCCATCA 3’  
Rv 5’ TCTCATGGCAGCTCTACACAC 3’ |
| G6Pase | Fw 5’ AACGTCTGTCTGCTCCGGATCTAC 3’  
Rv 5’ ACCTCTGGAGGCTCCATTG 3’ |
| S26 | Fw 5’ CGATTCTAGCAACCTTGCTATG 3’  
Rv 5’ CGTGCTCCTCCAAAGCTCTATG 3’ |

Fw = forward sequence; Rv = reverse sequence
Table S2: Effects of chronic intracerebroventricular infusion of Ang-(1-7) upon cardiovascular parameters in control-fed rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>MAP (mmHg)</th>
<th>HR (beats/ min)</th>
<th>BRS (ms/mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>5</td>
<td>112 ± 3</td>
<td>329 ± 9</td>
<td>1.06 ± 0.14</td>
</tr>
<tr>
<td>CTL+A7</td>
<td>5</td>
<td>112 ± 4</td>
<td>330 ± 8</td>
<td>0.93 ± 0.04</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. Group sizes are as n. Data were analyzed by Student t test. CTL- Control rats with ICV infusion of 0.9% sterile saline; CTL+A7- Control rats with ICV infusion of Ang-(1-7).
**Table S3:** Effect of chronic intracerebroventricular infusion of Ang-(1-7) upon body weight (BW), and relative weight of liver, retroperitoneal and epididymal adipose tissues, and left ventricle (LV) in fructose-fed rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>BW (g)</th>
<th>BW gain (g)</th>
<th>Liver (mg/g)</th>
<th>Retroperitoneal (mg/g)</th>
<th>Epididymal (mg/g)</th>
<th>LV (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>439 ± 9 (17)</td>
<td>228 ± 8 (12)</td>
<td>30 ± 0.5 (17)</td>
<td>7.4 ± 0.5 (15)</td>
<td>12.4 ± 0.7 (17)</td>
<td>2.1 ± 0.04 (13)</td>
</tr>
<tr>
<td>FF</td>
<td>436 ± 10 (13)</td>
<td>218 ± 7 (13)</td>
<td>33 ± 0.5 (13)*</td>
<td>9.5 ± 0.7 (13)*</td>
<td>12.8 ± 0.5 (13)</td>
<td>2.2 ± 0.02 (8)</td>
</tr>
<tr>
<td>FF+A7</td>
<td>448 ± 11 (12)</td>
<td>227 ± 7 (12)</td>
<td>31 ± 0.6 (11)†</td>
<td>10.5 ± 0.8 (12)*</td>
<td>13.6 ± 0.6 (12)</td>
<td>2.1 ± 0.05 (9)</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. Group sizes are in parentheses. *P < 0.05 vs CTL rats, †P < 0.05 vs FF rats. Data were analyzed by One-way ANOVA followed by Newman-Keuls post-hoc test. CTL- Control; FF- Fructose-fed with ICV infusion of 0.9 % sterile saline; FF+A7- Fructose-fed with ICV infusion of Ang-(1-7) rats.
Table S4: Effects of chronic intracerebroventricular infusion of Ang-(1-7) upon serum metabolic parameters in control-fed rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Glucose (mg/dL)</th>
<th>Insulin (ng/ml)</th>
<th>Leptin (ng/ml)</th>
<th>TGA (mg/dL)</th>
<th>T. Cholesterol (mg/dL)</th>
<th>HDL (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>113 ± 1.9 (9)</td>
<td>0.93 ± 0.12 (8)</td>
<td>2.3 ± 0.17 (9)</td>
<td>158 ± 12 (9)</td>
<td>62 ± 2 (9)</td>
<td>74 ± 3 (8)</td>
</tr>
<tr>
<td>CTL+A7</td>
<td>116 ± 1.6 (7)</td>
<td>1.10 ± 0.15 (5)</td>
<td>2.27 ± 0.35 (7)</td>
<td>162 ± 17 (7)</td>
<td>62 ± 3 (7)</td>
<td>75 ± 4 (5)</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. Group sizes are in parentheses. *P < 0.05 vs CTL rats. Data were analyzed by Student t test. TGA = triglycerides; T. Cholesterol = total cholesterol; HDL = high density lipoprotein. CTL- Control rats with ICV infusion of 0.9% sterile saline; CTL+A7- Control rats with ICV infusion of Ang-(1-7).
Table S5: Effect of chronic intracerebroventricular infusion of Ang-(1-7) upon mRNA expression of PEPCK and G6Pase in the liver of fructose-fed rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>PEPCK</th>
<th>G6Pase</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>13</td>
<td>1.00 (1.101-0.907)</td>
<td>1.00 (1.261-0.792)</td>
</tr>
<tr>
<td>FF</td>
<td>8</td>
<td>0.78 (0.952-0.653)</td>
<td>0.53 (0.702-0.400)</td>
</tr>
<tr>
<td>FF+A7</td>
<td>9</td>
<td>0.78 (0.914-0.681)</td>
<td>0.77 (0.948-0.632)</td>
</tr>
</tbody>
</table>

Values are fold change in mRNA expression in relation to the control group. Group sizes are as n. Data were analyzed by One-way ANOVA followed by Newman-Keuls post-hoc test. CTL- Control; FF- Fructose-fed with ICV infusion of 0.9% sterile saline; FF+A7- Fructose-fed with ICV infusion of Ang-(1-7) rats.
Figure S1. Changes in blood glucose after i.p. injection of D-glucose during glucose tolerance test of conscious control-fed rats with chronic ICV infusion of 0.9% sterile saline (CTL; $n=9$) and control-fed rats with chronic ICV infusion of Ang-(1-7) (CTL+A7; $n=7$). Values are mean ± SEM. Data were analyzed by Two-way ANOVA repeated measures followed by Student Newman-Keuls post-hoc test.