Peptides 37 (2012) 247–251

Contents lists available at SciVerse ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Short communication

Decreased hepatic gluconeogenesis in transgenic rats with increased circulating angiotensin-(1-7)

Victor Bilman[a], Lucas Mares-Guia[a], Ana Paula Nadu[a], Michael Bader[d], Maria José Campagnole-Santos[a], Robson Augusto S. Santos[a], Sérgio Henrique S. Santos[b,c,*]

[a] National Institute of Science and Technology (INCT-NanoBiofar), Physiology Department, Biological Sciences Institute (ICB), Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, MG, Brazil
[b] Pharmacology Department, Biological Sciences Institute (ICB), Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, MG, Brazil
[c] Laboratory of Health Science, Postgraduate Program in Health Sciences, UNIMONTES, Montes Claros, MG, Brazil
[d] Max-Delbrück-Center for Molecular Medicine (MDC), Berlin-Buch, Germany

ARTICLE INFO

Article history:
Received 6 July 2012
Received in revised form 1 August 2012
Accepted 1 August 2012
Available online 10 August 2012

ABSTRACT

The renin–angiotensin (Ang) system (RAS) plays an important role in the control of glucose metabolism and glycemia. Several studies demonstrated that the effects of angiotensin-(1-7) are mainly opposite to the actions of biological angiotensin II. Recent studies have demonstrated that rats with increased circulating angiotensin-(1-7), acting through the G protein coupled receptor Mas, have enhanced glucose tolerance and insulin sensitivity, presenting improved metabolic parameters. However, there is no data regarding the role of angiotensin-(1-7)–Mas axis in hepatic glycemic metabolism. In the present study, the gluconeogenesis and glycogenolysis was investigated in Sprague–Dawley (SD) and in TGR(A1-7)3292 (TGR) rats which present approximately twofold increase in plasma Ang-(1-7) levels compared to SD. The pyruvate administration in fasted rats showed a decreased synthesis of glucose in TGR compared to the SD rats, pointing to a downregulation of gluconeogenesis. Supporting this data, the mRNA evaluation of gluconeogenic enzymes showed a significant reduction in phosphoenolpyruvate carboxykinase reinforced by a significantly diminished expression of hepatocyte nuclear factor 4α (HNF-4α), responsible for the regulation of gluconeogenic enzymes. In conclusion our data show that the improved glucose metabolism induced by Ang-(1-7) could be due, at least in part, to a downregulation of hepatic gluconeogenesis.

1. Introduction

Glucose is the main energy source of the body and circulating glucose is derived from three sources: intestinal absorption during the fed state, in addition to glycogenolysis and gluconeogenesis during fasted states [1]. Gluconeogenesis takes place mainly in the liver, from precursors such as alanine and glutamine through pyruvate and finally glucose [6]. The HNF-4α gene, a hepatocyte nuclear factor, regulates the expression of genes responsible for gluconeogenic enzymes. Thus it plays an important role in this pathway and is considered a marker of gluconeogenesis [27].

The renin–angiotensin system (RAS) is now recognized to have a considerable influence in the development of cardiovascular and metabolic disorders. The RAS is composed of an enzymatic cascade in which angiotensinogen (AGT) is converted to Angiotensin (Ang) I by renin and subsequently to Ang II by angiotensin-converting-enzyme (ACE). Another important component of RAS, the Ang-(1-7), is primarily formed from Ang II by angiotensin converting enzyme 2 (ACE2). It is well documented that Ang II, acting via its AT_1 receptor, is a potent proinflammatory, pro-oxidant, and prothrombotic agent that interferes with several steps of intracellular insulin signaling. The ACE2/Ang-(1-7)/Mas axis has been suggested as an important counterregulatory arm in the RAS with opposite effects to those of ACE/Ang II/AT_1. The Ang-(1-7) can produce NO-dependent vasodilation as well as antiarrhythmic, antiproliferative, and antithrombotic effects [5,16,21–23].

Recently it was demonstrated that Mas-deficiency in FVB/N mice induces dyslipidemia, lower glucose tolerance and insulin sensitivity, hyperinsulinemia, hyperleptinemia, decreased glucose uptake in white adipose cells, in addition to an increase in adipose tissue mass. On the other hand, transgenic rats with increased circulating Ang-(1-7) (TGR) have improved lipid and glucose metabolism [22,23]. A recent study confirmed the increased Ang-(1-7) plasma levels in TGR (51.82 ± 6.3 in TGR vs. 29.17 ± 8.7 pg/mL in Sprague–Dawley rats); and also showed a lower body weight (278.3 ± 13.3 g in TGR vs. 375.7 ± 10.2 g in

* Corresponding author at: Pharmacology Department, Universidade Federal de Minas Gerais, Av Antonio Carlos 6627-ICB, 31270-901 Belo Horizonte, MG, Brazil. Tel.: +55 31 3409 2695/2724; fax: +55 31 3409 2695/2724.
E-mail address: sergiosousas@hotmail.com (S.H.S. Santos).

0196-9781/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.peptides.2012.08.002

Sprague–Dawley rats), improved insulin sensitivity and diminished triglycerides plasma levels (14.82 ± 3.77 mg/dL in TGR vs. 35.22 ± 3.39 mg/dL in Sprague–Dawley rats) in this model [23] However, the role of Ang-(1-7) in hepatic gluconeogenesis and glycogenolysis pathways is still poorly understood. Thus, the present study evaluated both pathways in the liver of transgenic rats which express Ang-(1-7) releasing fusion protein (TGR) showing approximately twofold increase in Ang-(1-7) plasma levels compared to Sprague–Dawley (SD) rats.

2. Materials and methods

2.1. Animals

Ten TGR and control Sprague–Dawley (SD) rats were obtained from the transgenic animal facilities at Laboratory of Hypertension (Federal University of Minas Gerais, Belo Horizonte, Brazil). The animals were kept under controlled light and temperature conditions, with free access to water and chow diet, in accordance to the ethical guidelines of our institution.

2.2. Tissue collection and blood measurements

Rats were sacrificed by decapitation and samples of blood and hepatic tissue were collected, weighed and immediately frozen in dry ice and stored at −80 °C for further analysis. Serum was obtained after centrifugation (3200 rpm for 10 min at 4 °C). ELISA kits were used to measure serum glucagon (ALPCO; Boston, USA) [10].

2.3. Hepatic glycogen content

Hepatic glycogen was extracted and determined as glucose following acid hydrolysis. Briefly, liver samples were placed in tubes with 30% KOH (Sigma; St. Louis, MO, USA) saturated with Na_2SO_4 (Sigma; St. Louis, MO, USA). The tubes were placed in a boiling water bath for 1 h until a homogeneous solution was obtained. Absolute ethanol was added to precipitate the glycogen from the alkaline digest. After centrifugation the supernatant was carefully aspirated and the glycogen washed. Glycogen precipitates were dissolved in 10 ml distilled water. The contents of the flasks were further diluted with water in a second volumetric flask so as to yield a solution of glycogen concentration of 3–30 mg/ml. Anthrone (Santa Cruz, CA, USA) was carefully added to 2 ml aliquots and the tubes were placed in boiling water. After the tubes cooled down, the absorbance of the samples was measured at 620 nm on a spectrophotometer. Glucose at different concentrations was used for a calibration curve [23].

2.4. Reverse transcription and real-time PCR

Total RNA from hepatic tissue was prepared using Trizol reagent (Invitrogen Corp., San Diego, CA, USA), treated with DNAse and reverse transcribed with M-MLV (Invitrogen Corp.) using random hexamer primers. Levels of glucose-6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase (PEPCK) and HNF4α mRNA were determined by real-time quantitative PCR using SYBR Green reagent (Applied Biosystems, CA, USA) in an ABI Prism 7000 platform (Applied Biosystems). The following primer pairs were used: glucose-6-phosphatase (G6Pase) forward 5_-aacgtctgtctgtcccggatctac-3_; G6Pase reverse 5_-acctctggaggctggcattg-3_; PEPCK forward 5_-tgcccatgcaaggcatca-3_; PEPCK reverse 5_-tctcatggcagctcctacaaacac-3_; hepatocyte nuclear factor 4 alpha (HNF4α) forward 5_-tgagcacctgctgcttgga-3_; HNF4α reverse 5_-tcgaggatgcgaatggacac-3_; β-actin forward 5_-tgacaggatgcagaaggaga-3_; β-actin reverse 5_-tagagccaccaatccacaca-3_ [23,27].

2.5. Western blotting analysis

Proteins were extracted from hepatic tissue samples (∼300 mg) of TGR and SD rats and 30 μg of protein were resolved on SDS-PAGE gels (10%) and then transferred onto nitrocellulose membranes. Glycogen phosphorylase enzyme, PYGB/L/M (Santa Cruz Biotechnology; CA, USA), and β-actin (internal control) (Cell Signaling Beverly; MA, USA) were probed with a polyclonal rabbit antibody (1:1000). Goat anti-rabbit IgG conjugated with peroxidase (1:5000) was used as a secondary antibody. The blots were visualized using a chemiluminescence western blotting detection reagent ECL; (Amersham Pharmacia Biotech, EUA) and revealed on a photographic film (Kodak; USA) followed by quantification using TINA 2.08c program (Raytest, Germany)

2.6. Glucagon and pyruvate challenge tests

For the serum glucagon measurement, glucagon extracted of porcine pancreas (0.2 mg/g of body weight) was intraperitoneally injected into overnight fasted rats. Glucose levels from tail blood samples were monitored at 0, 10, 20, 30, 60, 120, 150 and 180 min after injection using an Accu-Check glucometer (Roche Diagnostics Corp.; Indianapolis, IN, USA).

For pyruvate challenge test, fasted overnight rats were injected intraperitoneally with pyruvate (1 mg/g) as described by Sabio et al. [18]. Glucose levels from tail blood samples were monitored at 0, 10, 20, 30, 45, 60, 90 and 120 min after injection using an Accu-Check glucometer (Roche Diagnostics Corp.; Indianapolis, IN, USA) [11].

2.7. Statistical analysis

Data are expressed as the mean ± SEM. The statistical significance of difference in mean values between TGR and SD rats was assessed by unpaired Student's *t*-test or two-way ANOVA (glucagon and pyruvate challenge tests). Significance level was set at $p < 0.05$.

3. Results

Twelve weeks old TGR rats (0.0269 ± 0.00067 g/g BW) showed no difference in liver weight corrected by body weight when compared with SD rats. (0.0265 ± 0.00047 g/g BW) as illustrated in Fig. 1. Glucagon stimulation test also not demonstrate statistical difference between fasted TGR rats and SD rats (Fig. 2). Analysis of basal hepatic glycogen measurement showed no variation between TGR (0.4005 ± 0.1562 mg/g) and SD rats (0.5825 ± 0.1778 mg/g) as demonstrated in Fig. 2. In order to evaluate the gluconeogenesis pathway we performed the pyruvate challenge test (Fig. 1). Pyruvate administration in fasting TGR showed a decrease in the synthesis of glucose in these rats compared to the SD with the minimum peak for glycemic values of the curve in TGR rats at 30 min (106.8 in SD vs. 85.73 in TGR; $P < 0.01$) and 45 min (117.0 in SD vs. 98.00 in TGR; $P < 0.01$).

To understand the molecular mechanisms underlying changes in gluconeogenesis and glycogenolysis we analyzed the levels of glycongen phosphorylase enzyme, PYGB/L/M by Western blotting method (Fig. 2). The total of PYG enzyme level was not altered (4.148 ± 0.6282 in TGR vs. 5.893 ± 0.4164 in SD rats). In addition, real-time PCR analysis revealed a marked decrease in PEPCK expression in TGR hepatic tissue (1.403 ± 0.1441 in SD vs. 0.4598 ± 0.2391 in TGR), without difference in G6Pase expression in TGR and SD rats (0.7363 ± 0.09964 in SD vs. 1.133 ± 0.2475 in TGR) as showed in Fig. 1. In order to confirm the downregulation in gluconeogenesis we evaluated the mRNA expression of HNF-4α, responsible for the regulation of transcription enzymes on gluconeogenesis pathway (Fig. 1), and we observed an important

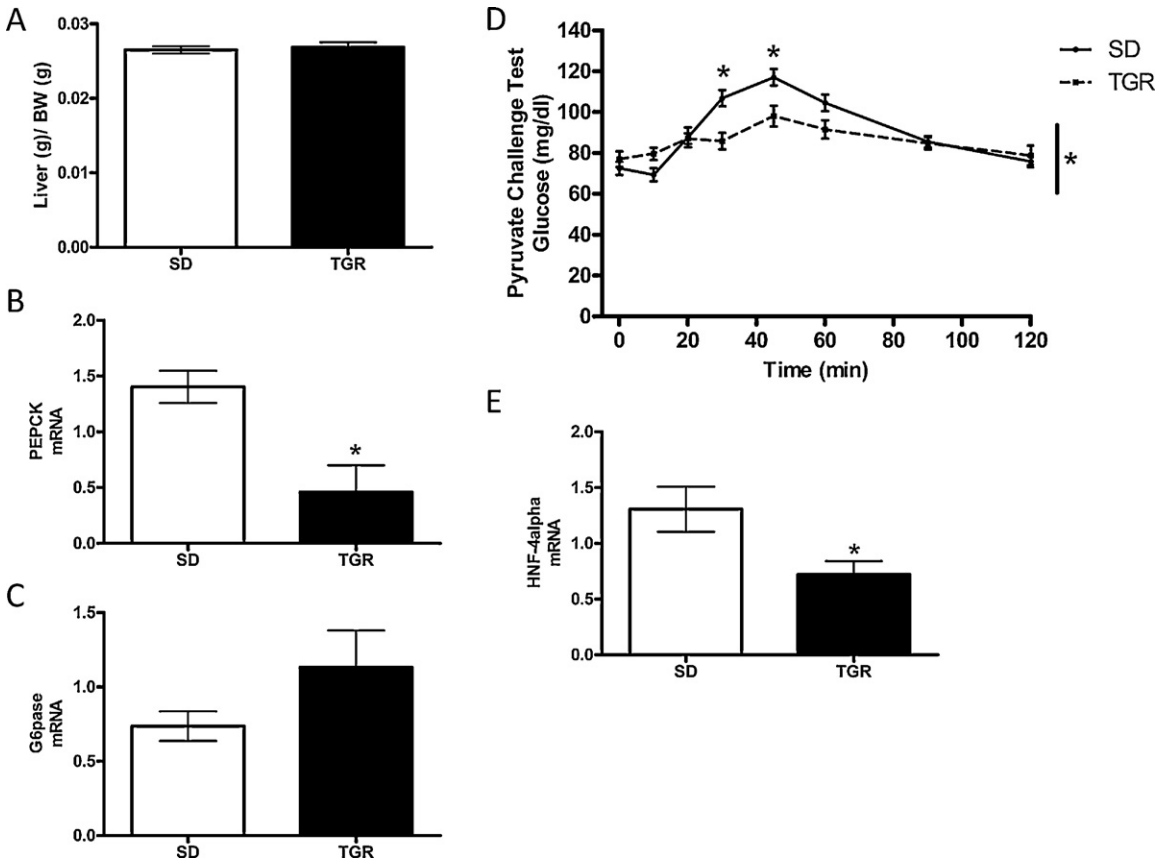

Fig. 1. (A) Liver weight corrected by body weight (g) of 12 weeks-old TGR ($n = 6$) and SD ($n = 6$) male rats. (B) Expression of PEPCK. (C) Expression of G6Pase. (D) Pyruvate challenge test. Overnight-fasted rats were given an intraperitoneal injection of pyruvate (1 mg/g body weight). Blood samples were collected from the tail at indicated times and analyzed for glucose concentration. (E) Expression of HNF-4α. Data are presented as means ± SE. $^*P < 0.05$.

decrease in TGR rats (0.7214 ± 0.1196 in TGR vs. 1.307 ± 0.2023 in SD).

4. Discussion

It is well documented that Ang-(1-7) presents several effects opposite to those produced by Ang II [13,15,20,22,23], however, this is the first study evaluating the role of Ang-(1-7) on liver gluconeogenesis and glycogenolysis. The main result of the present study was to show that transgenic rats with increased circulating Ang-(1-7) presents a decreased activation of the gluconeogenesis pathway, demonstrated by the pyruvate challenge test accompanied by a significantly reduction in PEPCK and HNF4α.

The role of Ang II in glucose metabolism is well established. Coimbra et al. [4] demonstrated that administration of Ang II increases hepatic glucose output, mostly by activation of gluconeogenesis pathway in comparison to the glycogenolysis pathway. The present results point to a counterregulatory action of Angiotensin-(1-7) on gluconeogenesis, which opposes the effect of Ang II.

Gluconeogenesis process is controlled directly or indirectly by many hormones, including insulin and glucagon [7]. The glucagon stimulation test was performed in fasted rats and no significant difference was observed between fasted TGR and SD rats. However, this result can be attributed to the action of glucagon in all the metabolic sensitive tissues of the rat (such as muscle) and not exclusively in the liver.

Glycogenolysis was evaluated through baseline hepatic glycogen concentration and levels of hepatic glycogen phosphorylase, an allosteric enzyme responsible for catalyzing the phosphorylation of glycogen to glucose1-P, playing a fundamental role in glycogen metabolism [7,26]. There was no significant difference in hepatic glycogen phosphorylase levels analyzed by Western blotting. The absence of alteration in glycogenolysis pathway can explain the unaltered hepatic glycogen levels in TGR.

To evaluate gluconeogenesis pathway separately we performed the pyruvate challenge test [18]. The first regulated step in the gluconeogenic pathway from pyruvate and its precursors is the pyruvate to oxaloacetate carboxylation, catalyzed by ATP-dependent pyruvate carboxylase [8–10]. The pyruvate challenge experiment showed that overnight fasted TGR rats have a decrease in the glucose synthesis when compared to overnight fasted SD rats, suggesting a downregulation in the gluconeogenesis pathway, since overnight fasted rats have negligible amounts of preformed glycogen.

In order to confirm the downregulation of the gluconeogenesis pathway, it was evaluated the mRNA expression of the key enzymes of this route. The expression of G6Pase, a multicomponent enzyme system that hydrolyses glucose-6-phospate (G6P) to glucose in the final step of gluconeogenesis, showed no statistically difference in TGR and SD rats. PEPCK, one of the main rate-limiting enzymes of gluconeogenesis, simultaneously decarboxilates and phosphorylates oxaloacetate to phosphoenolpyruvate, had its expression significantly reduced in TGR when compared to SD rats. These results suggest that the gluconeogenesis downregulation could be due to the decreased expression of PEPCK.

Recently, it has been documented that HNF4α has been implicated in gluconeogenesis through transcriptional regulation of G6Pase and PEPCK, which are rate-limiting enzymes in this process as discussed previously [27]. The mRNA expression of HNF4α analysis by RT-PCR showed significantly decreased levels

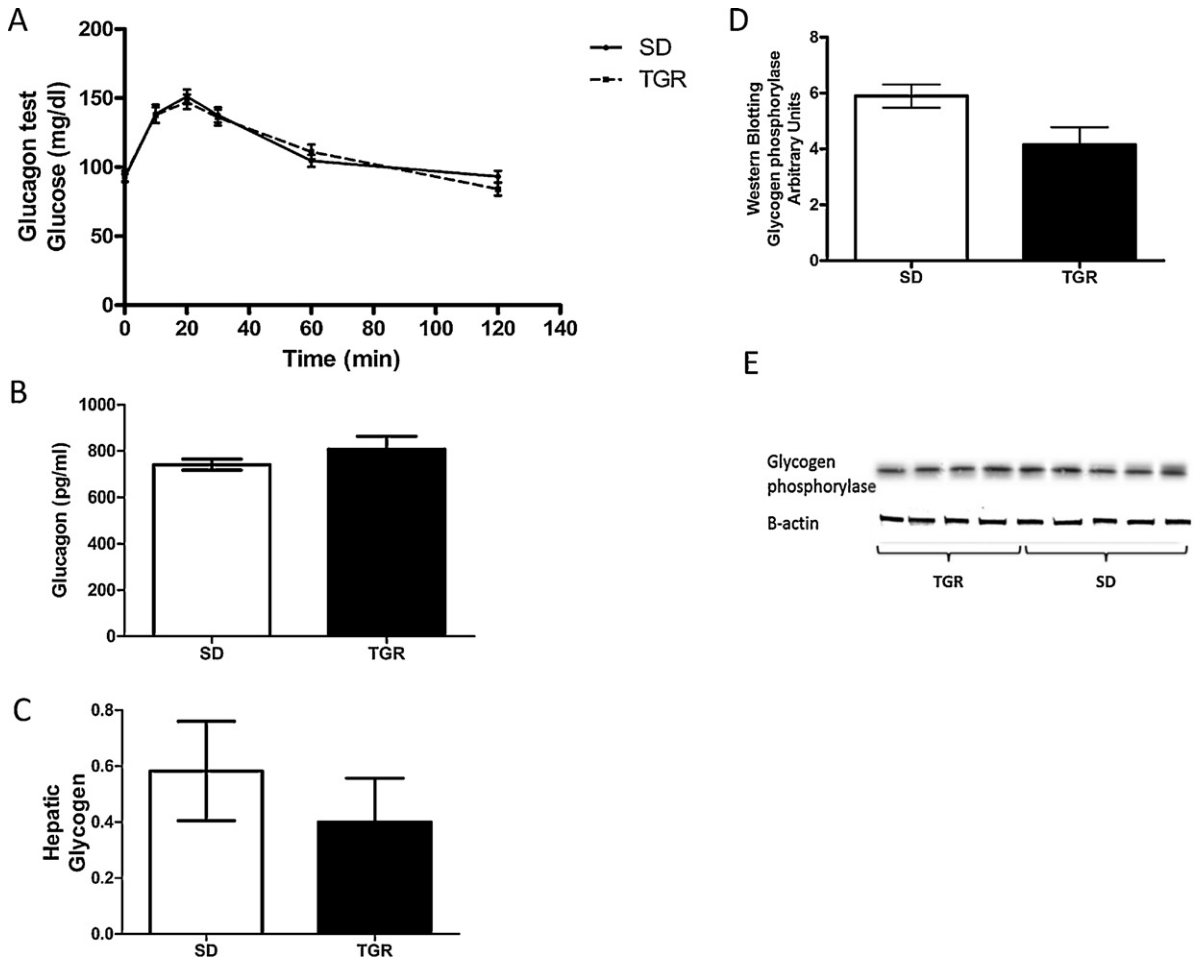

Fig. 2. (A) Glucagon stimulation test. Overnight-fasted rats were given an intraperitoneal injection of glucagon (0.2 mg/g body weight). (B) Glucagon serum levels. (C) Levels of basal hepatic glycogen. (D) Glycogen phosphorylase Western blotting analyses of hepatic tissue in TGR rats ($n = 4$) and Sprague–Dawley (SD) ($n = 5$) rats. (E) Illustrative picture of G6Pase Western blotting gel from TGR and SD rats. After incubation with primary and secondary antibody for glycogen phosphorylase detection, the membrane was stripped and incubated with primary and secondary antibody used to detect β-actin. Data are presented as means ± SE. No statistically significant differences between the groups were observed.

in TGR, when compared to SD rats. This finding pointed out to a relation between Ang-(1-7) and HNF4α, leading to an overall downregulation of gluconeogenesis. This result can be responsible, at least in part, for the improved circulating glycemic profile in TGR described previously [23].

In summary, the results obtained in the present study show that transgenic rats with increased Ang-(1-7) plasma levels, present a lower activation of the gluconeogenesis pathway responsible for glucose synthesis, without evidence of alteration in the hepatic glycogenolysis. This result is evidenced by the lower peaks of plasma glucose levels in fasted TGR rats during pyruvate challenge test. The primary mechanisms involved in this effect appear to include a decrease in hepatocyte nuclear factor 4α (HNF-4α) expression, probably leading to a down regulation of PEPCK, one of the main rate-limiting enzymes of gluconeogenesis. These findings suggest an important role of Ang-(1-7) in hepatic glucose metabolism.

Acknowledgments

This work was supported by a grant of CNPq (INCT-NanoBiofar), FAPEMIG, PRONEX (FAPEMIG/CNPq-Edital 17/2010) and CAPES. There are none competing of interests.

References

[1] Aronoff SL, Berkowitz K, Schreiner B, Want L. Glucose metabolism and regulation: beyond insulin and glucagon. Diabetes Spectrum 2004;17: 183–90.
[4] Coimbra CC, Garofalo MA, Foscolo DR, Xavier AR, Mogliorini RH. Gluconeogenesis activation after intravenous angiotensin II in freely moving rats. Peptides 1999;20:823–7.
[5] Ferreira AJ, Santos RAS. Cardiovascular actions of angiotensin-(1-7). Braz J Med Biol Res 2005;38:499–507.
[6] Gray S, Wang B, Orihuela Y, Hong EG, Fisch S, Haldar S, et al. Regulation of gluconeogenesis by Kruppel-like factor 15. Cell Metab 2007;5:305–12.
[7] Heppner KM, Habegger KM, Day J, Pfluger PT, Perez-Tilve D, Ward B, et al. Glucagon regulation of energy metabolism. Physiol Behav 2010;100(5):545–8.
[8] Jitrapakdee S, Booker GW, Cassady AI, Wallace JC. Cloning, sequencing and expression of rat liver pyruvate carboxylase. Biochem J 1996;316(Pt. 2):631–7.
[9] Jitrapakdee S, Booker GW, Cassady AI, Wallace JC. The rat pyruvate carboxylase gene structure. Alternate promoters generate multiple transcripts with the 54-end heterogeneity. J Biol Chem 1997;272:20522–30.
[10] Jitrapakdee S, St Maurice M, Rayment I, Cleland WW, Wallace JC, Attwood PV. Structure, mechanism and regulation of pyruvate carboxylase. Biochem J 2008;413:369–87.
[11] Jones CG, Titheradge MA. The effect of treatment of the rat with bacterial endotoxin on gluconeogenesis and pyruvate metabolism in subsequently isolated hepatocytes. Biochem J 2003;289:169–72, physbeh.2010.03.019.
[13] Lemos VS, Cortes SF, Silva DM, Campagnole-Santos MJ, Santos RAS. Angiotensin-(1-7) is involved in the endothelium-dependent modulation of phenylephrine induced contraction in the aorta of mRen-2 transgenic rats. Br J Pharmacol 2002;135:1743–8.

[15] Lovren F, Pan Y, Quan A, Teoh H, Wang G, Shukla PC, et al. Angiotensin converting enzyme-2 confers endothelial protection and attenuates atherosclerosis. Am J Physiol Heart Circ Physiol 2008;295:H1377–84.
[16] Mario EG SS, Ferreira AV, Bader M, Santos RA, Botion LM. Angiotensin-(1-7) Mas-receptor deficiency decreases peroxisome proliferator-activated receptor gamma expression in adipocytes. Peptides 2012;33:174–7.
[18] Sabio G, Das M, Mora A, Zhang Z, Jun JY, Ko HJ, et al. A stress signaling pathway in adipose tissue regulates hepatic insulin resistance. Science 2008;322: 1539–43.
[20] Santos RAS, Ferreira AJ, Simões e Silva AC. Recent advances in the angiotensin-converting enzyme 2-angiotensin(1-7)-Mas axis. Exp Physiol 2008;93(5):519–27.
[21] Santos RA, Simões e Silva AC, Maric C, Silva DM, Machado RP, de Buhr I, et al. Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. Proc Natl Acad Sci USA 2003;100(14):8258–63.
[22] Santos SH, Fernandes LR, Mario EG, Ferreira AV, Porto LC, Alvarez-Leite JI, et al. Mas deficiency in FVB/N mice produces marked changes in lipid and glycemic metabolism. Diabetes 2008;57:340–7.
[23] Santos SH, Mario BJ, Pôrto EG, Rodrigues-Machado Mda LC, Murari G, Botion A, et al. Improved lipid and glucose metabolism in transgenic rats with increased circulating angiotensin-(1-7). Arterioscler Thromb Vasc Biol 2012;30: 953–61.
[26] Vardanega-Peicher Márcia, Galleto Ricardo, Pagliarini e Silva Sarah, Bazzote Roberto Barbosa. Comparative effect of glucagon and isoproterenol on hepatic glycogenolysis and glycolysis in isolated perfused liver. Braz Arch Biol Technol 2003;46(4):563–8.
[27] Yasui K, Miyoshi PNN, Suzuki T, Taguchi K, Ishigami Y, Fukutomi R, et al. Effects of a catechin-free fraction derived from green tea on gene expression of enzymes related to lipid metabolism in the mouse liver. Biomed Res 2012;33(1):9–13.