Chronic infusion of angiotensin-(1–7) improves insulin resistance and hypertension induced by a high-fructose diet in rats

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Submitted 5 August 2008; accepted in final form 8 November 2008

Chronic infusion of angiotensin-(1–7) improves insulin resistance and hypertension induced by a high-fructose diet in rats. Am J Physiol Endocrinol Metab 296: E262–E271, 2009. First published November 11, 2008; doi:10.1152/ajpendo.90678.2008.—The current study was undertaken to determine whether Ang-(1–7) is effective in improving metabolic parameters in fructose-fed rats (FFR), a model of metabolic syndrome. Six-week-old male Sprague-Dawley rats were fed either normal rat chow (control) or the same diet plus 10% fructose in drinking water. For the last 2 wk of a 6-wk period of either diet, control and FFR were implanted with subcutaneous osmotic pumps that delivered Ang-(1–7) (100 ng·kg−1·min−1). A subgroup of each group of animals (control or FFR) underwent a sham surgery. We measured systolic blood pressure (SBP) together with plasma levels of insulin, triglycerides, and glucose. A glucose tolerance test (GTT) was performed, with plasma insulin levels determined before and 15 and 120 min after glucose administration. In addition, we evaluated insulin signaling through the IR/IRS-1/PI3K/Akt pathway as well as the phosphorylation levels of IRS-1 at inhibitory site Ser307 in skeletal muscle and adipose tissue. FFR displayed hypertriglyceridemia, hyperinsulinemia, increased SBP, and an exaggerated release of insulin during a GTT, together with decreased activation of insulin signaling through the IR/IRS-1/PI3K/Akt pathway in skeletal muscle, liver, and adipose tissue, as well as increased levels of IRS-1 phospho-Ser307 in skeletal muscle and adipose tissue, alterations that correlated with increased activation of the kinases mTOR and JNK. Chronic Ang-(1–7) treatment resulted in normalization of all alterations. These results show that Ang-(1–7) ameliorates insulin resistance in a model of metabolic syndrome via a mechanism that could involve the modulation of insulin signaling.

serine phosphorylation; renin-angiotensin system; insulin signaling
Glucose, triglycerides, and insulin measurements. All corresponding measurements were determined 6 h after food removal. Blood glucose measurements were performed using a hand-held glucometer (Accuchek, Mannheim, Germany). Insulin levels were assessed using a rat insulin ELISA kit (Ultra Sensitive Rat Insulin ELISA Kit; Crystal Chem). Circulating triglyceride (TG) concentrations were measured by an enzymatic colorimetric assay kit (Wiener Lab, Rosario, Argentina).

The homeostasis model assessment of basal insulin resistance (HOMA-IR) was used to calculate an index from the product of the fasting concentrations of plasma glucose (mmol/l) and plasma insulin (μU/ml) divided by 22.5 (31). Lower HOMA-IR values indicated greater insulin sensitivity, whereas higher HOMA-IR values indicated lower insulin sensitivity (insulin resistance).

Glucose tolerance test. After a 6-h fast the rats were anesthetized, and after the collection of an unchallenged sample (time 0), a solution of 50% glucose (2.0 g/kg body wt) was administered into the peritoneal cavity. During the test, blood was collected from the tail vein 15, 30, 60, 90, and 120 min after glucose administration. All blood glucose measurements were performed using a hand-held glucometer. Serum insulin levels were measured at baseline and 15 and 120 min postglucose administration by ELISA, as described above.

Acute insulin stimulation and IRS-1 phosphorylation. Therefore, after the 2-wk treatment with Ang-(1–7), rats were starved overnight, anesthetized by the intraperitoneal administration of a mixture of ketamine and xylazine (50 and 1 mg/kg, respectively), and submitted to the surgical procedure as soon as anesthesia was assured by the loss of pedal and corneal reflexes. The abdominal cavity was opened, the portal vein was exposed, and 10 IU porcine insulin/kg body wt in normal saline (0.9% NaCl) in a final volume of 0.2 ml was injected via this vein. To obtain data under basal conditions, rats received an injection of diluent. The liver, adipose tissue (epididymal), and skeletal muscle (soleus) were removed after 1, 3, and 5 min, respectively, and kept at −80°C until analysis.

Tissue homogenization, immunoprecipitation, Western blotting analysis, and quantification of phosphorylation of IRS-1 at Ser632 by ELISA. Tissue samples were homogenized in solubilization buffer containing 1% Triton together with phosphatase and protease inhibitors, as described previously (17, 33). Tissue extracts were centrifuged at 100,000 g for 1 h at 4°C to eliminate insoluble material, and protein concentration in the supernatants was measured using the Bradford protein assay. The proteins were denatured by boiling in reducing buffer, resolved by SDS-PAGE, and transferred to nitrocellulose membranes. After blocking, membranes were incubated with the appropriate phospho-specific antibodies (1:1,000 dilution). Protein abundance was detected by reprobing membranes with the corresponding antibodies (1:200 or 1:1,000). Membranes were subsequently incubated overnight with the anti-phosphotyrosine antibody (1:1,000) to detect tyrosine phosphorylation. Membranes were reblotted with anti-IR (1:200) or anti-IRS-1 (1:1,000) to determine protein abundance of these two proteins. To determine the amount of the p85 subunit of PI3K associated with IRS-1, membranes corresponding to anti-IRS-1 immunoprecipitates were also reprobed with anti-p85 antibody (1:2,000). To determine the phosphorylation of IRS-1 at Ser632, IRS-1 immunoprecipitates were probed with the anti-phospho-IRS-1 (Ser632) antibody (1:1,000). To determine the phosphorylation levels of Akt, JNK, mTOR, or IKK, equal amounts of solubilized protein (2 mg) were incubated at 4°C overnight with anti-IR or anti-IRS-1 antibodies at a final concentration of 4 μg/ml. Immune complexes were collected by incubation with protein A-Sepharose 6 MB, as described previously (17, 33). SDS-PAGE and Western transfer of proteins to PVDF membranes were performed as described previously (17, 33). Membranes were blocked by incubation for 2 h with Tris-buffered saline containing 0.1% Tween-20 and 3% BSA and subsequently incubated overnight with the anti-phosphotyrosine antibody (1:1,000) to detect tyrosine phosphorylation. Membranes were reblotted with anti-IR (1:200) or anti-IRS-1 (1:1,000) to determine protein abundance of these two proteins. To determine the amount of the p85 subunit of PI3K associated with IRS-1, membranes corresponding to anti-IRS-1 immunoprecipitates were also reprobed with anti-p85 antibody (1:2,000). To determine the phosphorylation of IRS-1 at Ser632, IRS-1 immunoprecipitates were probed with the anti-phospho-IRS-1 (Ser632) antibody (1:1,000). To determine the phosphorylation levels of Akt, JNK, mTOR, or IKK, equal amounts of solubilized proteins (40 μg) were denatured by boiling in reducing sample buffer, resolved by SDS-PAGE, and subjected to immunoblotting with the corresponding phospho-specific antibodies (1:1,000 dilution). Protein abundance was detected by reprobing membranes with the corresponding antibodies. After extensive washing, membranes were incubated with the appropriate secondary HRP-coupled antibodies and processed for ECL using the ECL plus Western Blotting detection system (Amersham Biosciences). Bands were quantified using Gel-Pro Analyzer 4.0 (Media Cybernetics, Bethesda, MD). IRS-1 phospho-Ser632 levels were measured in aliquots of homogenates containing 0.2 mg of protein using a commercial solid-
RESULTS

Metabolic characteristics of the experimental animals and effect of Ang-(1–7) treatment. At the end of 6-wk high-fructose diet feeding, body weights were similar between standard chow-fed and fructose-fed rats (Table 1). As expected, on the basis of published data in the fructose-fed rat model (9, 32, 41), circulating insulin and TG were significantly higher in the fructose-fed rats (88% of mean fructose-fed values, \( P < 0.05 \)) compared with the control sham group, indicative of an amelioration of the insulin-resistant state after chronic treatment with Ang-(1–7) (Table 1).

Ang-(1–7) improves insulin sensitivity in fructose-fed rats. The response to an intraperitoneal glucose tolerance test (IPGTT) at the termination of 2-wk treatment with Ang-(1–7) is shown in Fig. 1. Plasma glucose levels increased significantly during the IPGTT in all groups of animals. Compared with the control sham group, the fructose-fed group of rats presented a tendency to an increase in the glucose area under the curve during the IPGTT (Fig. 1). However, this change did not reach statistical significance \( (P = 0.1) \). Treatment with Ang-(1–7) did not exert a significant influence on glucose tolerance in either control or fructose-fed rats (Fig. 1).

Table 1. Metabolic parameters of the experimental animals

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control Sham</th>
<th>Ang-(1–7) Sham</th>
<th>Fructose</th>
<th>Ang-(1–7) Fructose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>380 ± 9</td>
<td>362 ± 9</td>
<td>385 ± 12</td>
<td>375 ± 7</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>112 ± 14</td>
<td>116 ± 4</td>
<td>135 ± 7*</td>
<td>120 ± 4</td>
</tr>
<tr>
<td>Fasted glycemia, mg/dl</td>
<td>112 ± 9</td>
<td>109 ± 9</td>
<td>114 ± 10</td>
<td>112 ± 11</td>
</tr>
<tr>
<td>Plasma insulin, ng/ml</td>
<td>1.2 ± 0.2</td>
<td>1.6 ± 0.2</td>
<td>2.8 ± 0.4*</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>82 ± 13</td>
<td>71 ± 11</td>
<td>176 ± 18*</td>
<td>115 ± 12</td>
</tr>
<tr>
<td>HOMA score</td>
<td>2.8 ± 0.9</td>
<td>3.1 ± 0.9</td>
<td>7.9 ± 1.7*</td>
<td>3.9 ± 0.7</td>
</tr>
</tbody>
</table>

Values are means ± SE \( (n = 12 \) animals in each group). Ang-(1–7), angiotensin-(1–7); SBP, systolic blood pressure; HOMA, homeostasis model assessment. \*Value significantly different from control sham value \( (P < 0.05) \).

Plasma insulin concentrations were measured at baseline and 15 and 120 min after glucose injection. Circulating insulin concentrations were significantly larger in fructose-fed rats compared with the control group at baseline (2.4-fold increase, \( P < 0.05 \)) 15 min after injection (2.2-fold increase, \( P < 0.05 \)) and remained significantly higher after 120 min (2.1-fold increase, \( P < 0.05 \)). This altered insulin secretory response would suggest a state of insulin resistance in the fructose-fed rats. The insulin secretory response was sensibly reduced in fructose-fed rats after Ang-(1–7) treatment compared with their sham counterparts, indicative of an amelioration of the insulin-resistant state after chronic treatment with Ang-(1–7). This
result is in line with the observation of reduced circulating TG and insulin after infusion with Ang-(1–7). Treatment with Ang-(1–7) for 14 days did not significantly modify the release of insulin during the IPGTT in the sham-implanted animals fed a regular diet (Fig. 1).

Angiotensin-(1–7) improves insulin signal transduction in skeletal muscle, adipose tissue, and liver. Fructose feeding is known to produce an impairment of insulin signaling in rats (6, 30). In this study we evaluated insulin signaling through the IR/IRS-1/Pi3K/Akt pathway in insulin target tissues of rats fed on regular chow or with fructose in drinking water. An impairment in all steps of insulin signaling analyzed IR (Fig. 2), IRS-1 (Fig. 3), and p85-IRS-1 association (Fig. 4), and Akt phosphorylation (Fig. 5) was detected in skeletal muscle, adipose tissue, and liver of fructose-fed rats. To determine the effect of chronic Ang-(1–7) on insulin signal transduction, Sprague-Dawley rats fed with 10% fructose on drinking water were treated with Ang-(1–7) for 14 days and evaluated for insulin signaling in the tissues described above. As shown in Figs. 2–5, significant improvement in insulin signal transduction through the steps that result in activation of Akt was detected in skeletal muscle, adipose tissue, and liver of rats treated with Ang-(1–7). As detected by specific immunoblotting, changes in phosphorylation were not the consequence of changes in the abundance of the signaling proteins analyzed (Figs. 2–5).

Treatment of fructose-fed rats with Ang-(1–7) is associated with decreased phosphorylation of IRS-1 on Ser307 and decreased activation of the kinases JNK and mTOR in skeletal muscle and white adipose tissue. To address the mechanism involved in the improvement of insulin signaling and action in fructose-fed rats originated by treatment with Ang-(1–7), we measured the levels of IRS-1 phosphorylation at the inhibitory residue Ser307 in peripheral insulin target tissues muscle and white adipose tissue. As shown in Fig. 6, compared with values found in control rats, there was a significant increase in IRS-1 Ser307 phosphorylation in rats fed with fructose in both skeletal muscle (35% as measured by ELISA and 120% as measured by immunoblotting, \( P < 0.05 \) in both cases) and white adipose tissue (22% as measured by ELISA and 126% as measured by immunoblotting, \( P < 0.05 \) in both cases). Treatment of fructose-fed rats with Ang-(1–7) was found to reduce IRS-1 Ser307 phosphorylation to control values in both tissues, whereas it did not induce significant changes in control animals (Fig. 6). This modulation of phospho-IRS-1 Ser307 was independent of modulation of total IRS-1 levels (Fig. 6).

To further evaluate this mechanism of inhibition of the insulin-signaling pathway in fructose-fed rats as well as the impact of Ang-(1–7) treatment, we evaluated the phosphorylation status of JNK, mTOR, and IKK\( \beta \), three kinases involved in the signaling pathway responsible for the phosphorylation of IRS-1 upon Ser307. As shown in Fig. 7, fructose-fed rats showed a significant increase in the phosphorylation levels of JNK and mTOR in skeletal muscle (increases of 94 and 202%, respectively, \( P < 0.05 \) vs. control rats) and adipose tissue (increases of 47 and 203%, respectively, \( P < 0.05 \) vs. control rats). In contrast, the phosphorylation levels of IKK\( \beta \) remained unaltered after fructose treatment.

The protein abundance of the kinases analyzed was unaltered by the fructose treatment (Fig. 7, bottom).
DISCUSSION

The major finding of this study was that chronic Ang-(1–7) treatment resulted in a reversal of fructose-induced insulin resistance. The favorable effects of chronic Ang-(1–7) treat-

Fig. 3. p-Tyr of insulin receptor substrate (IRS-1). Rats were injected via portal vein with porcine insulin. The skeletal muscle (A), adipose tissue (epididymal; B), and liver (C) were removed 5, 3, and 1 min, respectively, after injection, and p-Tyr was measured by specific IB in IRS-1 IP. To determine IRS-1 abundance, membranes were reprobed with an anti-IRS-1 antibody. Quantification of phosphorylation by scanning densitometry is given below IB. Scanning data obtained from 6 independent experiments are expressed as fold increase over saline-injected (basal) control phosphorylation ± SE. *P < 0.05 vs. insulin-stimulated control values.

Fig. 4. Association between the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) and IRS-1. Rats were injected via portal vein with porcine insulin. The skeletal muscle (A), adipose tissue (epididymal; B), and liver (C) were removed 5, 3, and 1 min, respectively, after injection, and the amount of p85 was measured by specific immunoblotting in IRS-1 IP. Quantification of p85 associated with IRS-1 by scanning densitometry is given below IB. Scanning data obtained from 6 independent experiments are expressed as fold increase over saline-injected (basal) control p85-IRS-1 association ± SE. *P < 0.05 vs. insulin-stimulated control values.

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ment in insulin-resistant animals included reduction of fasting triglyceride and insulin levels, reduction of systolic blood pressure, and restoration of insulin signaling through the IR/IRS-1/PI3K/Akt pathway in the main target tissues of insulin: skeletal muscle, liver, and adipose tissue. Together with recent demonstration that genetic deletion of Mas in FVB/N mice leads to a metabolic syndrome-like state (43), our present findings indicate that the Mas/Ang-(1–7) axis could participate in glycemic control.

The RAS is known to play an important role in insulin resistance and diabetes, Ang II being an important mediator of adverse processes (38, 39). On the other hand, Ang-(1–7) appears to exert a protective role in diabetes. Recent reports indicate that Ang-(1–7) attenuates proteinuria, protects the heart in response to ischemia/reperfusion, and restores the normal function of the renal vasculature in diabetic rats (4, 5). We have shown that Ang-(1–7) modulates the insulin-signaling pathway (17), suggesting that this beneficial effect of Ang-(1–7) could be the result of such modulation. This putative new physiological function of Ang-(1–7) was the subject of exploration of the current study.

The fructose-fed rat model is a commonly used environmentally induced model that mimics human metabolic syndrome in many aspects, including hypertension, hypertriglyceridemia, insulin resistance, and compensatory hyperinsulinemia. Fructose-induced metabolic syndrome can be created experimentally by either adding fructose to drinking water (10–20%) (9, 32, 41) or feeding rats with a high-fructose diet (60%) (6, 30).

In the present study, we looked at Ang-(1–7) on insulin resistance induced by administration of 10% fructose in drinking water, which proved to induce a prediabetic state, as shown by the need for augmented insulin release to maintain euglycemia in the face of a glucose challenge. Previous studies have shown that circulating Ang II levels are increased in fructose-fed rats (29). In addition, administration of both Ang II receptor antagonists (15, 25, 28, 36) or ACE inhibitors (13, 23) has been shown to revert the insulin resistance in fructose-fed rats, strongly suggesting that Ang II is involved in the insulin-resistant state developed by fructose overload. In view of these data, and given that Ang-(1–7) counteracts most of Ang II actions, we hypothesize that, in part, our current observation showing reversal of insulin resistance in fructose-fed rats after Ang-(1–7) treatment could be the result of a counterbalance exerted by Ang-(1–7) on the deleterious effects of Ang II with regard to lipid and carbohydrate metabolism.

It is worth mentioning that chronic treatment with Ang-(1–7) using the same dose and methodology employed in the current study has previously proven to have a therapeutic effect. Grobe et al. (18) demonstrated that delivery of Ang-(1–7) prevents Ang II-induced cardiac remodeling. In addition, these authors demonstrated that chronic treatment with Ang-(1–7) prevents cardiac fibrosis in rats treated with deoxycorticosterone acetate (19).

The mechanism behind the amelioration of insulin resistance and improvement of insulin signaling induced by Ang-(1–7) in fructose-fed rats is intriguing and deserves further exploration. One potential mechanism involved could be the ability of Ang-(1–7) to activate, unlike Ang II, the enzyme Akt, a phenomenon that has been described in both endothelial cells and in the heart and is associated with Mas receptor activation (17, 40). It is important to consider that, at least in the heart,
Ang-(1–7) antagonizes the inhibitory effects of Ang II on insulin-induced activation of Akt (17). Thus, the observed improvement in insulin signaling after Ang-(1–7) treatment could be related to this effect exerted by Ang-(1–7). Hemodynamic effects with improved delivery of insulin and glucose to peripheral tissues could also be involved in the beneficial effects induced by Ang-(1–7) in fructose-fed rats. Ang-(1–7) is a vasodilator, in part due to its recently demonstrated capability of activating the endothelial nitric oxide synthase (40) and also because of its proven ability to potentiate the action of bradykinin (26). Gestational diabetes is associated with decreased levels of Ang-(1–7), which coincides with endothelial dysfunction during pregnancy (35). This agrees with the beneficial effects (vasodilation, improvement of glucose, and lipid metabolism) exerted by Ang-(1–7).

Among the serine residues of the IRS-1 that become phosphorylated in response to risk factors for insulin resistance, Ser307 has been studied extensively and has become a molecular indicator of insulin resistance (1, 2, 24). Phosphorylation of IRS-1 at this site in skeletal muscle and adipose tissue is increased in insulin-resistant states (11). IRS-1 is phosphorylated at Ser307 by multiple kinases, including JNK (1, 24), mTOR (7), and IKK (16). Accordingly, in our current study, we have investigated the phosphorylation of IRS-1 Ser307 and shown that fructose-fed rats display an increase in IRS-1 Ser307 phosphorylation levels together with increased activation of the serine kinases JNK and mTOR in skeletal muscle and adipose tissue. Moreover, we demonstrated that improvement of insulin signaling associated with Ang-(1–7) treatment is accompanied with decreased phosphorylation at this inhibitory residue and

Fig. 6. Phosphorylation of IRS-1 on Ser307. Data are from saline-injected rats, as described in the legend to Fig. 1. Phospho (p)-Ser307 levels were measured in tissue homogenates (A: skeletal muscle; B: adipose tissue) by the use of a specific ELISA (a) or by means of IP of tissue proteins with an anti-IRS-1 antibody followed by IB with an anti-phospho-IRS-1 (Ser307) antibody (b). To determine protein abundance, membranes were reprobed with the anti-IRS-1 antibody. Data are means ± SE (n = 6). *P < 0.05 vs. all other groups.
**Fig. 7.** Phosphorylation and abundance of inhibitor of NF-κB kinase (IKKβ), JNK, and mammalian target of rapamycin (mTOR). Data are from saline-injected rats, as described in the legend to Fig. 1. Phosphorylation of IKKβ, mTOR, JNK or was measured by specific IB. Membranes were reprobed with specific anti-peptide antibody to determine protein abundance. Quantification of phosphorylation by scanning densitometry is given below IB. Scanning data obtained from 6 independent experiments are expressed as fold increase over saline-injected (basal) control phosphorylation ± SE. *P < 0.05 vs. insulin-stimulated control values.
decreased activation levels of JNK and mTOR in skeletal muscle and adipose tissue of fructose-fed rats. This finding suggests an additional mechanism by which Ang-(1–7) exerts beneficial effects on the insulin-signaling system.

In conclusion, in the current study we provide novel data suggesting that Ang-(1–7) ameliorates insulin resistance in a model of metabolic syndrome via a mechanism that could involve the modulation of insulin signaling and particularly the modulation of the mTOR pathway involved in phosphorylation of IRS-1 at Ser residue 307. Together with the recently reported phenotype of Mas-knockout receptor mice (43), our current findings reinforce the notion that Ang-(1–7) possesses a role in metabolic processes.

GRANTS
F. P. Dominici, M. M. Gironacci, C. A. Taira, and D. Turyn are Career Investigators from Consejo Nacional de Investigaciones Científicas y Tecnológicas of Argentina (CONICET) and received grant support from the University of Buenos Aires (UBA), CONICET, and Agencia Nacional de Promoción Científica y Tecnológica of Argentina. J. F. Giani is a research fellow from UBA; M. A. Mayer and M. C. Muñoz are research fellows from CONICET.

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