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The Mas receptor mediates modulation of insulin signaling by angiotensin-(1–7)

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ABSTRACT

Angiotensin (Ang)-(1–7) stimulates proteins belonging to the insulin signaling pathway and ameliorates the Ang II negative effects at this level. However, up to date, receptors involved and mechanisms behind these observations remain unknown. Accordingly, in the present study, we explored the *in vivo* effects of antagonism of the Ang-(1–7) specific Mas receptor on insulin signal transduction in rat insulin-target tissues. We evaluated the acute modulation of insulin-stimulated phosphorylation of Akt, GSK-3β (Glycogen synthase kinase-3β) and AS160 (Akt substrate of 160 kDa) by Ang-(1–7) and/or Ang II in the presence and absence of the selective Mas receptor antagonist A-779 in insulin-target tissues of normal rats. Also using A-779, we determined whether the Mas receptor mediates the improvement of insulin sensitivity exerted by chronic Ang-(1–7) treatment in fructose-fed rats (FFR), a model of insulin resistance, dyslipidemia and mild hypertension. The two major findings of the present work are as follows; 1) Ang-(1–7) attenuates acute Ang II-mediated inhibition of insulin signaling components in normal rats via a Mas receptor-dependent mechanism; and 2). The Mas receptor appears to be involved in beneficial effects of Ang-(1–7) on the phosphorylation of crucial insulin signaling mediators (Akt, GSK-3β and AS160), in liver, skeletal muscle and adipose tissue of FFR. These results shed light into the mechanism by which Ang-(1–7) exerts its positive physiological modulation of insulin actions in classical metabolic tissues and reinforces the central role of Akt in these effects.

1. Introduction

Alterations within the renin-angiotensin system (RAS) are an important contributor to the development of insulin resistance [1–3]. The RAS is classically conceived as a coordinated hormonal cascade in the control of cardiovascular, renal, and adrenal functions, mainly through the actions of angiotensin (Ang) II [4]. Advances in cell and molecular biology have led to the recognition of other active fragments of RAS metabolism, such as Ang III, Ang IV, and Ang-(1–7), the angiotensin-converting enzyme (ACE) 2, an homolog of classic ACE that forms Ang-(1–7) directly from Ang II and indirectly from Ang I [5], and the Ang-(1–7) specific G protein-coupled receptor Mas [6]. In general terms, the ACE2/Ang-(1–7)/Mas receptor axis opposes the vascular and proliferative effects of Ang II [7].

A large body of evidence indicates that Ang II plays a critical role in the etiology of insulin resistance [8]. The mechanism behind this deleterious effect appears to be related to a negative modulation exerted by Ang II through AT1 receptor (AT1R) on several steps of the insulin signaling cascade, including insulin-induced phosphorylation of the insulin receptor, insulin receptor substrate-1, and activation of Akt mediated by a mechanism that involves phosphatidylinositol 3-kinase (PI3K) [8]. Accordingly, clinical trials have shown that inhibition of ACE or selective AT1R blockade reduces the development of type 2 diabetes in patients with essential hypertension [9,10]. In line with reports in humans, improvement of insulin sensitivity [1,11–13] along with an enhancement in the response to insulin at various steps of the insulin signaling cascade [14–16], has been detected in various animal models of insulin resistance and/or type 2 diabetes as a consequence of reduction of Ang II formation or inhibition of its actions. This evidence clearly indicates that the signaling crosstalk between insulin and Ang II has significant physiological relevance. In contrast, Ang-(1–7), through its specific G protein-coupled receptor Mas induces responses that oppose those of Ang II, including antihypertensive, antihypertrophic, antifibrotic and antithrombotic properties [5,7]. Considering that inhibition of ACE or chronic blockade of AT1R is associated with increased levels of circulating Ang-(1–7), this hormone could be involved in the beneficial effects of antihypertensive therapy [17–19].

We have previously demonstrated that Ang-(1–7) reverses insulin resistance in rats fed a high-fructose diet [20]. This effect could be ascribed to the capability of Ang-(1–7) to induce the activation of Akt in insulin-target tissues [21,22] and also to counteract the inhibitory effects of Ang II on insulin-induced Akt phosphorylation [22]. In line with the finding that Ang-(1–7) has metabolic actions, Santos

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et al. reported that genetic deletion of the specific Ang-(1–7) Mas receptor leads to a metabolic syndrome-like state in mice [23]. More recently, it was reported that rats overexpressing Ang-(1–7) display improved glucose and lipid metabolism [24], and that ACE2 gene therapy improves glycemic control in diabetic mice, through a mechanism mediated by Ang-(1–7) acting through its specific receptor Mas [25]. Despite this available information, the mechanism behind the amelioration of insulin resistance induced by Ang-(1–7) through its specific receptor Mas in FFR is intriguing and deserves further exploration.

Accordingly, in the current study, we explored the effects of simultaneous acute injection of different combinations of Ang-(1–7) and/or Ang II in the presence and absence of the selective Mas receptor antagonist A-779 on insulin-stimulated phosphorylation of Akt, GSK-3β (Glycogen synthase kinase-3β) and AS160 (Akt substrate of 160 kDa) in insulin-target tissues of normal animals. In addition, by the use of A-779, we determined the role of the Mas receptor in the improvement of insulin sensitivity exerted by chronic Ang-(1–7) treatment in FFR, a model of insulin resistance, dyslipidemia and mild hypertension.

2. Methods

2.1. Reagents

The peptides Ang II, Ang-(1–7) and [^{7}-D-Ala-Ang-(1–7)] (A-779) were purchased from Bachem Americas, Inc. (Torrance, CA, USA.). The reagents and apparatus for SDS-PAGE and immunoblotting were obtained from Bio-Rad (Hercules, CA, USA). The phospho-Akt (Ser473) rabbit polyclonal antibody that detects endogenous levels of Akt only when phosphorylated at Ser473 (anti-p-Akt-Ser473; 9271), the rabbit polyclonal Akt antibody that detects endogenous levels of total Akt1, Akt2 and Akt3 proteins (anti-Akt; 9272), the phospho-GSK-3β rabbit polyclonal antibody that detects endogenous levels of glycogen synthase kinase 3β only when phosphorylated at Ser9 (anti-p-GSK-3β-Ser9; 9336), the monoclonal antibody that detects total levels of GSK-3β (anti-GSK-3β; 9315) and the phospho-AS160 (Thr642) rabbit polyclonal antibody (anti-p-AS160-Thr642; 4288) and the anti-AS160 antibody (anti-AS160; 2670) were purchased from Cell Signaling (Beverly, MA, USA). The polyclonal antibody that detects total levels of Mas receptor was purchased from Alomone Labs (Jerusalem, Israel). The polyclonal goat anti-rabbit IgG conjugated with Horse Radish Peroxidase (HRP) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Enhanced chemiluminescence (ECL) was purchased from GE Healthcare (Piscataway, NJ, USA). The remaining reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Protocol 1. Analysis of the *in vivo* interactions between Ang II, Ang-(1–7) and insulin in liver, muscle and adipose tissue of normal animals.

2.2. Animals

A total of 24 male Sprague–Dawley rats weighing 220–240 g were used for this study. Animals were housed in a controlled environment with a photoperiod of 12 h light–12 h dark (lights on from 06:00 to 18:00 h) and a temperature of 20 ± 2 °C. Sanitary controls were performed for all major rodent pathogens and the results of these tests were uniformly negative. Animals were given free access to water and nutritionally balanced diet (16%–18% protein; Cargill, Argentina). Housing, handling and experimental procedures followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocols were approved by the Animals Studies Committee of the School of Pharmacy and Biochemistry, University of Buenos Aires, Argentina.

2.3. Surgical procedures, hormone administration and tissue homogenization

Rats were starved overnight, anesthetized by the intraperitoneal (i.p) administration of a mixture of ketamine and xylasine (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, and an *in vivo* acute stimulation of all tissues analyzed was obtained by the injection of 200 μl solutions containing either normal saline (0.9% NaCl), Ang II (8 pmol/kg), Ang-(1–7) (8 pmol/kg) or insulin (8 pmol/kg) or simultaneously with insulin and Ang II, with insulin and Ang-(1–7), or with a combination of these three hormones into the portal vein. For selective antagonism of the Mas receptor, A-779 (80 pmol/kg) was administered intravenously together with a combination of these three hormones ($n = 3$ per group). In a previous work, we demonstrated that Ang-(1–7) induces the *in vivo* phosphorylation of Akt in the heart, which peaks 5 min after injection of an 8 pmol/kg dose of the hormone [21]. Thus, we decided to perform the experiment within 5 min after the stimulation with solutions containing different compositions for all tissues with the exception of the liver. The injection into the portal vein ensures a rapid arrival of these compounds into the liver. In a time course analysis (data not shown), we verified that Ang-(1–7) induced the *in vivo* phosphorylation of Akt in the liver, which peaked 1–2 min after injection of an 8 pmol/kg dose of the hormone. Accordingly, liver, adipose tissue (epididymal) and skeletal muscle (*soleus*) were removed 1, 3 and 5 min after injection respectively, and kept at −80 °C until analysis. Tissue samples were homogenized in 10 volumes of a solubilization buffer containing 1% Triton together with phosphatases and protease inhibitors as described previously [21]. All tissues extracts were centrifuged at 100,000 ×*g* for 1 h at 4 °C to eliminate insoluble material, and protein concentration in the supernatants was determined using the bicinchoninic acid method [26].

2.4. Western blotting analysis

To determine the phosphorylation levels of Akt (Ser473), GSK-3β (Ser9), AS160 (Thr642) and the corresponding total protein abundance, equal amounts of solubilized proteins (40 μg) in Laemmli buffer were resolved by SDS-PAGE and subjected to immunoblotting with anti-phospho-Akt, anti-phospho-GSK-3β, anti-phospho-AS160, anti-Akt, anti-GSK-3β or anti-AS160 (1:3000 dilutions for all antibodies). After washing, membranes were incubated for 1 h with goat anti-rabbit IgG-HRP secondary antibody (1:20,000 dilution), proteins detected by ECL and the intensities of the specific bands were quantitated by optical densitometry. The blots shown are representative of four different experiments. Protein loading in gels was evaluated with an anti-tubulin antibody.

Protocol 2. Analysis of the role of the Mas receptor in the Ang-(1–7)-induced reversal of insulin resistance in fructose-fed rats.

2.5. Animals and treatments

A total of 30 male Sprague–Dawley rats weighing 220–240 g were used for this study. All animals were housed individually in a controlled environment with a photoperiod of 12 h light–12 h dark (lights on from 06:00 to 18:00 h) and a temperature of 20 ± 2 °C. Housing, handling and experimental procedures followed the rules written in the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health [DHEW Publication No. (NIH) 85-23, Revised 1996, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205]. Following an acclimatization period of 7 days, rats received regular diet (19% protein, 77% carbohydrate, 4% fat) and fructose that was administered as a 10% solution (prepared every 2 days) in drinking water during 6 weeks as described

previously [20]. For the last 2 weeks of the high fructose feeding period, the animals were divided in 4 groups: FFR-control group ($n=6$). FFR with Ang-(1–7) [FFR-Ang-(1–7)] group ($n=6$). FFR with A-779 [FFR-A-779] group ($n=6$) and FFR with Ang-(1–7) and A-779) [FFR-Ang-(1–7)/A-779] group ($n=6$). Animals were implanted with subcutaneous osmotic pumps (model 2002, Alzet, CA) that delivered Ang-(1–7) (100 ng · kg^{-1} · min^{-1}), A-779 (500 ng · kg^{-1} · min^{-1}) or a mixture of Ang-(1–7) and A-779 (100 ng · kg^{-1} · min^{-1} /500 ng · kg^{-1} · min^{-1}) respectively. The FFR-control group ($n=6$) underwent a sham surgery.

2.6. Systolic blood pressure and body weight determination

Rats were weighed previously to dietary manipulation and at the end of the study. The rats were trained to the procedure of systolic blood pressure (SBP) measurement at 1300 h, twice a week, for 2 weeks previous to the final measurement. The mean of ten consecutive readings was used as the reported value of the systolic blood pressure for each rat. Indirect SBP was measured at week 4 and week 6 by means of the tail-cuff method using a blood pressure analysis system (model SC1000, Hatteras Instruments, North Carolina, USA).

2.7. Glucose, triglycerides and insulin measurements

All determinations were performed 6 h after food removal. Blood glucose measurements were performed using a hand-held glucometer (Accucheck, Mannheim, Germany). Insulin levels were assessed using a rat insulin ELISA kit (Ultra Sensitive Rat Insulin ELISA Kit; Crystal Chem, Inc). Circulating triglycerides (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 [27]. Lower HOMA-IR values indicated greater insulin sensitivity, whereas higher HOMA-IR values indicated lower insulin sensitivity (insulin resistance).

2.8. Acute insulin stimulation and tissue collection

After the 2 week treatment with Ang-(1–7), A-779 or both compounds, rats were starved overnight, anesthetized by the i.p. administration of a mixture of ketamine and xylasine (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 of porcine insulin per kg body weight in normal saline (0.9% NaCl) in a final volume of 0.2 ml was injected. To obtain data under basal conditions, rats received an injection of vehicle. 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.

2.9. Tissue homogenization and western blotting analysis

This analysis was performed as described for Protocol 1.

2.10. Immunohistochemical staining

Paraffin sections (sagital) were cut at 3 μm, dewaxed in xylene, rehydrated through a series of descending concentrations of alcohol to water, and treated with 3% hydrogen peroxide for 30 min to inhibit endogenous peroxidase. Local Ang II was detected using a rabbit polyclonal antibody anti-Ang II (Phoenix Pharmaceutical, Inc, Burlingame, CA, USA) at a 1:100 dilution using a previously described protocol [14]. Sections were treated with biotinylated horse anti-rabbit/mouse/goat IgG (1:200) for 30 min at room temperature and incubated with the avidin-biotin-peroxidase complex (Vector, Burlingame, CA) for 60 min. Peroxidase activity was visualized by exposing the sections for 1 min to 3,30-diaminobenzidine tetrahydrochloride (Vector, Burlingame, CA) in PBS containing 3% H_2O_2. Sections were then counterstained with hematoxylin. In the negative controls, the primary antibody was replaced by nonimmune serum. Four tissues sections from each experimental animal were stained. On each section, ten consecutive microscopic fields (400× magnification) were analyzed to evaluate Ang II immunostaining density in all tissues analyzed. All histological sections were studied in each animal using a light microscope (Leica Microsystems, Wetzlar, Germany).

2.11. Statistical analysis

All values are reported as means ± S.E.M unless specified otherwise. Significance, considered as $P<0.05$, was determined by analysis of variance (ANOVA) followed by the Tukey–Kramer test using GraphPad InStat version 5.00 for Windows by GraphPad Software, Inc. (San Diego, CA, USA).

3. Results

3.1. Protocol 1: in vivo interactions between Ang II, Ang-(1–7) and insulin on the phosphorylation of Akt, GSK-3β and AS160 in liver, muscle and adipose tissue

As shown in Fig. 1, upper panel, insulin (8 pmol/kg) stimulated the phosphorylation of Akt in liver, adipose tissue and skeletal muscle. The acute administration of Ang II alone (8 pmol/kg) did not stimulate phosphorylation of Akt at Ser473. On the other hand, Ang-(1–7) induced the phosphorylation of Akt to a similar extent of that attained after stimulation with insulin.

Insulin-induced phosphorylation of Akt was blunted in the presence of Ang II (Fig. 1, upper panel). The simultaneous administration of a mixture of insulin, Ang II and Ang-(1–7) at equivalent doses resulted in a similar level of phosphorylation of Akt to that achieved after acute administration of insulin alone. A similar result was obtained after co-administration of insulin and Ang-(1–7) (Fig. 1, upper panel). Similar changes were detected for the phosphorylation of Akt at Thr308 (data not shown). The total amount of Akt protein analyzed was not affected by the different treatments, as confirmed by submitting the corresponding total tissues extracts to immunoblotting with anti-Akt (Fig. 1, middle panel).

Similarly, compared to basal values, GSK-3β Ser9 phosphorylation increased significantly in rats that received insulin, Ang-(1–7) or a mixture of both hormones, (Fig. 2, upper panel). When injected alone, Ang II did not stimulate increase GSK-3β phosphorylation. In addition, insulin failed to stimulate GSK-3β phosphorylation when co-administered with Ang II (Fig. 2, upper panel). As shown in Fig. 2, middle panel, hormone treatment did not affect the total amount of GSK-3β in any condition or tissue analyzed. Finally, we explored the activation of a novel described substrate of Akt of 160 kDa (AS160), that is required for GLUT-4 translocation in skeletal muscle and adipose tissue. As observed in Fig. 3, in all tissues analyzed, both insulin and Ang-(1–7) stimulated the phosphorylation of AS160 to a similar extent. Acute administration of Ang II alone did not stimulate Thr phosphorylation of AS160, while insulin-induced phosphorylation of AS160 was blunted in the presence of Ang II (Fig. 3, upper panel). In line with results obtained for Akt and GSK-3β, insulin-induced phosphorylation of AS160 was blunted in the presence of Ang II (Fig. 3, upper panel). On the other hand, the administration of a mixture of insulin, Ang II and Ang-(1–7) in the same dose, resulted in a similar level of phosphorylation of AS160 to that achieved after acute administration of insulin alone, indicating that Ang-(1–7) counteracted the negative modulation of insulin signaling exerted by Ang II (Fig. 3, upper panel). Ang-(1–7) did not enhance the insulin stimulation of AS160 (Fig. 3, upper panel). The total amount of AS160 protein analyzed was not affected by the different treatments, as confirmed by submitting the corresponding total skeletal muscle

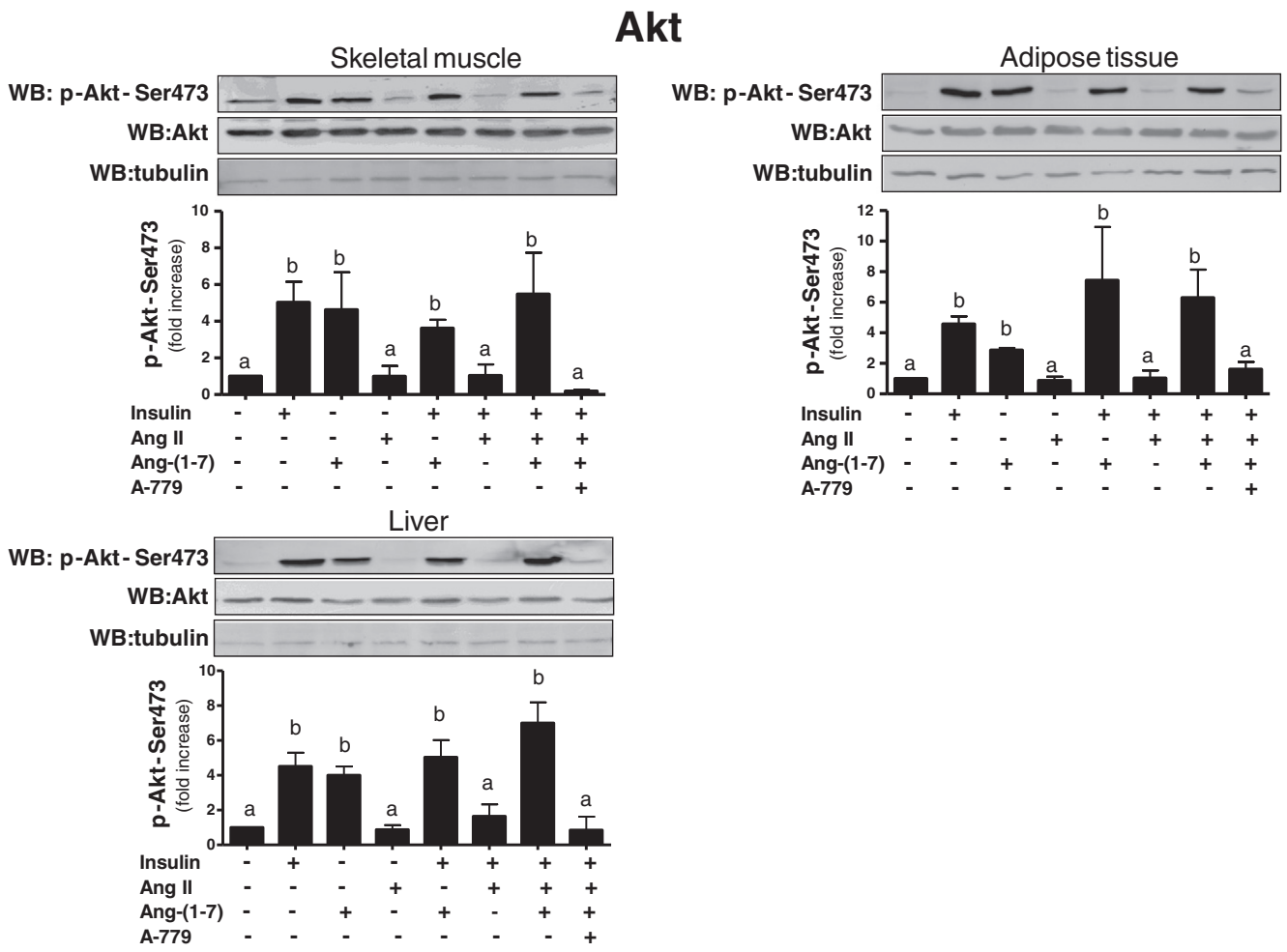

Fig. 1. Protocol 1: Akt-Ser473 phosphorylation in skeletal muscle, adipose tissue and liver of normal rats. Animals were anesthetized and acutely treated with a single i.v dose (0.2 ml via portal vein) of a solution of normal saline (−) or solutions of normal saline containing insulin (8 pmol/kg), Ang II (8 pmol/kg), Ang-(1–7) (8 pmol/kg) or their combinations as indicated. For selective antagonism of the Mas receptor, A-779 (80 pmol/kg) was administered intravenously together with a combination of these three hormones. The liver, adipose tissue (epididymal) and skeletal muscle (*soleus*) were removed 1, 3 and 5 min after injection respectively. Solubilized tissue proteins were subjected to immunoblotting with anti-phospho-Akt (p-Akt-Ser473) (upper panels). To determine protein abundance, total tissue extracts were subjected to western blotting with anti-Akt antibodies (lower panels). Bar graphs show the quantitative Akt-Ser473 phosphorylation. Data (means ± SEM) are expressed as fold increases in phosphorylation over basal ($n = 3$). In each group, values marked with a different superscript (a or b) are significantly different between them ($P < 0.05$). WB: western blotting.

and adipose tissue extracts to immunoblotting with a specific anti-AS160 antibody (Fig. 3, middle panel).

To confirm the participation of the Mas receptor in the Ang-(1–7)-induced phosphorylation of Akt, GSK-3β and AS160, a mixture of insulin, Ang II and Ang-(1–7) was co-administered with the Mas receptor antagonist A-779. As shown in Figs. 1, 2 and 3, upper panels, the presence of A-779 blocked the stimulating effects of Ang-(1–7) on both the phosphorylation of Akt and its downstream substrates GSK-3β and AS160 respectively. Protein loading in gels was evaluated with an anti-tubulin antibody (lower panels).

3.2. Protocol 2: Ang II immunostaining in liver, skeletal muscle and adipose tissue of FFR

As shown in Fig. 4, fructose-fed control rats exhibited extensive areas of Ang II immunostaining in liver, skeletal muscle and adipose tissue. No significant differences were observed in the immunostaining for Ang II in any tissue analyzed.

3.3. Protocol 2: Mas receptor levels in liver, skeletal muscle and adipose tissue of FFR

The Ang-(1–7) specific receptor Mas in liver, skeletal muscle and adipose tissue from FFR was analyzed by Western Blot. As shown in Fig. 5 all groups of experimental animals displayed similar levels of Mas receptor abundance in liver and skeletal muscle. However, treatment with Ang-(1–7) induced a significant increase in the abundance of the Mas receptor in adipose tissue (Fig. 5, upper panel).

3.4. Protocol 2: Ang-(1–7)-induced modulation of insulin sensitivity in FFR is mediated by a Mas receptor-dependent pathway

At the end of the study, all groups of rats analyzed showed similar body weight and plasma glucose levels (Table 1). After two weeks treatment with Ang-(1–7), FFR display lower SBP together with a significant reduction in both serum insulin and triglycerides compared to FFR treated with saline only ($P < 0.05$). Treatment with Ang-(1–7) also caused a significant decrease in the HOMA score in FFR (Table 1). Specific

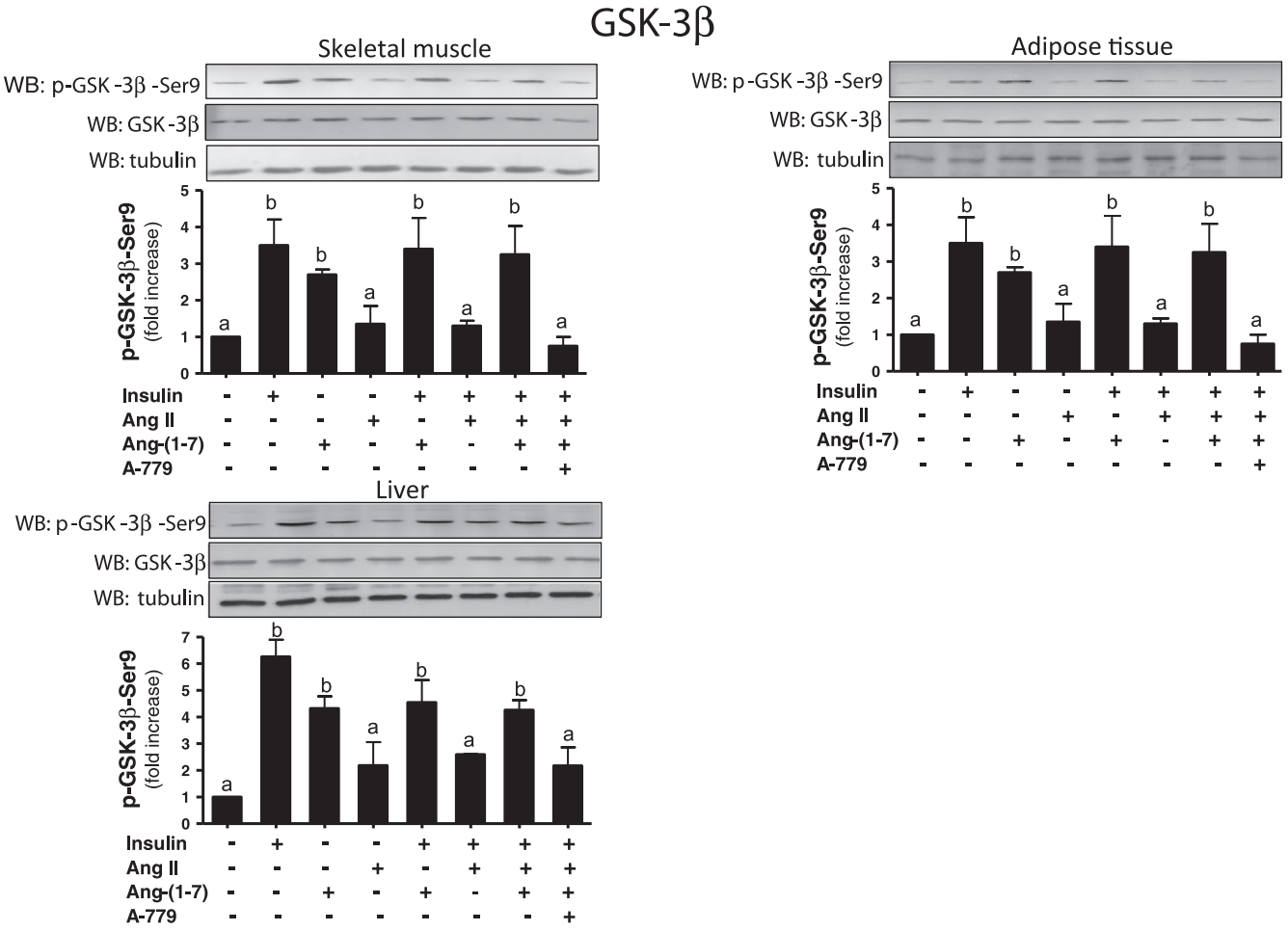

Fig. 2. Protocol 1: GSK-3β-Ser9 phosphorylation in skeletal muscle, adipose tissue and liver of normal rats. Rats were treated as described for Fig. 1. At the indicated time points, the liver, adipose tissue (epididymal) and skeletal muscle (soleus) were removed and homogenized as described in Methods. Solubilized tissue proteins were subjected to immunoblotting with anti-phospho-GSK-3β (p-GSK-3β-Ser9) (upper panels). To determine protein abundance, total tissue extracts were subjected to western blotting with anti-GSK-3β antibodies (lower panels). Bar graphs show the quantitative GSK-3β-Ser9 phosphorylation. Data (means ± SEM) are expressed as fold increases in phosphorylation over basal ($n = 3$). In each group, values marked with a different superscript (a or b) are significantly different between them ($P < 0.05$). WB: western blotting.

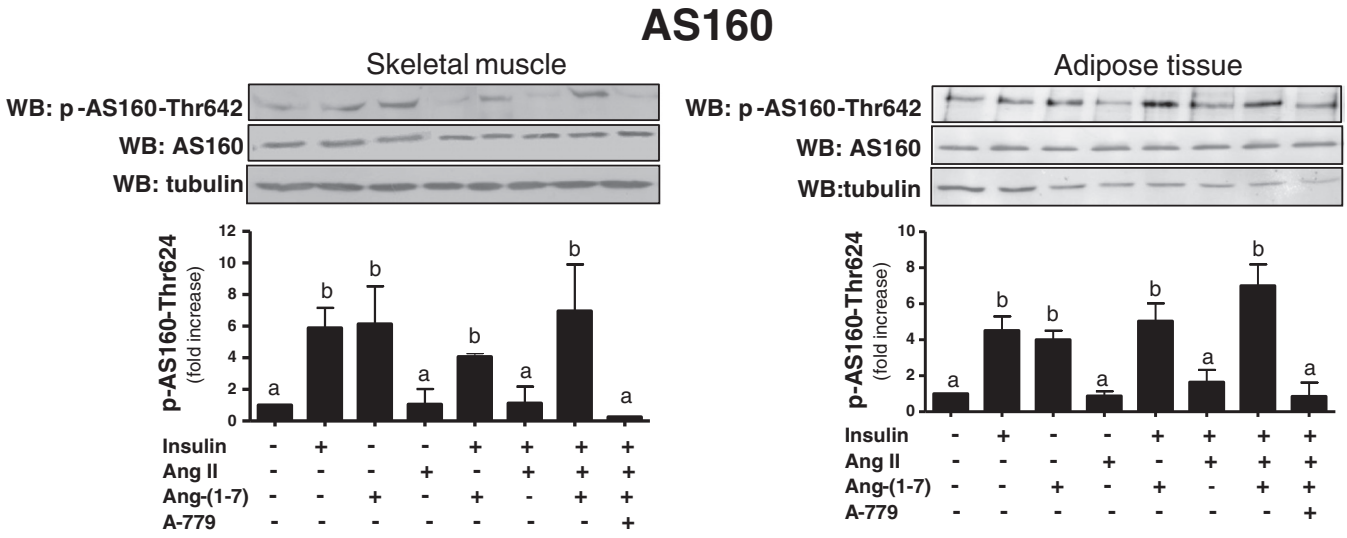

Fig. 3. Protocol 1: AS160-Thr642 phosphorylation in skeletal muscle and adipose tissue of normal rats. Rats were treated as described for Fig. 1. At the indicated time points, the liver, adipose tissue (epididymal) and skeletal muscle (soleus) were removed and homogenized as described in Methods. Solubilized tissue proteins were subjected to immunoblotting with anti-phospho-AS160 (p-AS160-Thr642) (upper panels). To determine protein abundance, total tissue extracts were subjected to western blotting with anti-AS160 antibodies (lower panels). Bar graphs show the quantitative AS160-Thr642 phosphorylation. Data (means ± SEM) are expressed as fold increases in phosphorylation over basal ($n = 3$). In each group, values marked with a different superscript (a or b) are significantly different between them ($P < 0.05$). WB: western blotting.

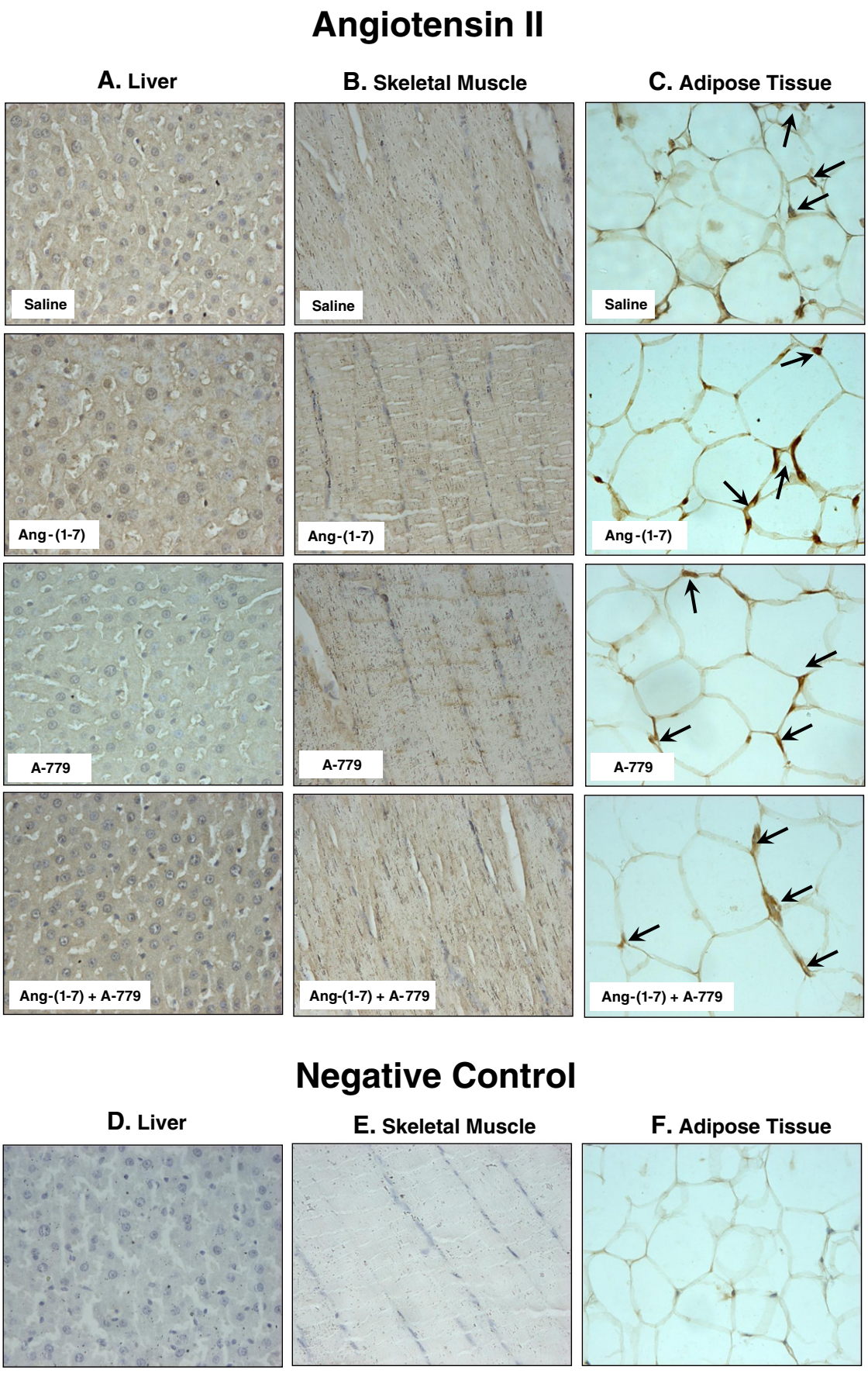

Fig. 4. Protocol 2: Representative sections showing positive immunostaining for Ang II in liver, skeletal muscle and adipose tissue from FFR-saline, FFR + Ang-(1–7), FFR + A-779 and FFR + Ang-(1–7) + A-779 (original magnification ×400). Sections show extended areas of positive staining for Ang II in liver (A), skeletal muscle (B) and adipose tissue (C) from all groups analyzed. Tissue sections from FFR groups were incubated with nonimmune serum (D, E, F).

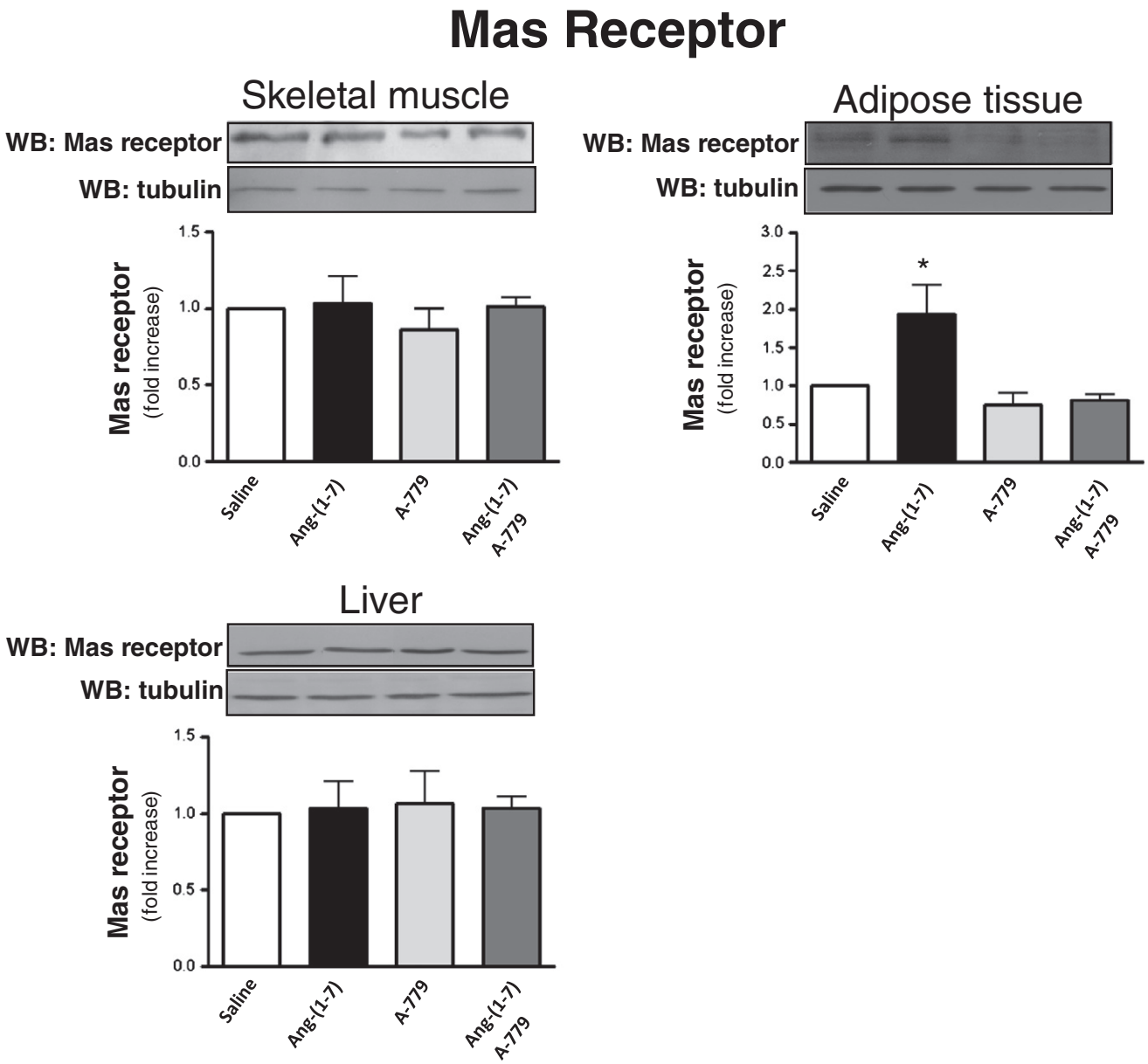

Fig. 5. Protocol 2: Mas receptor abundance in skeletal muscle, adipose tissue and liver of fructose-fed rats. Tissue homogenates were submitting to western blot analysis. Representative images and bar charts showing the quantification of Mas receptor for each group. Data (means ± SEM) are expressed as fold increases in Mas abundance over basal ($n = 6$; *$P < 0.05$). WB: western blotting.

antagonism of the Mas receptor with A-779 blocked the beneficial effects of Ang-(1–7) on SBP and metabolic parameters (Table 1). When compared with untreated FFR, chronic administration to FFR of the Mas antagonist alone did not modify any of the metabolic parameters analyzed in these animals (Table 1).

Table 1
Metabolic parameters of the experimental animals.

Parameters	Saline	Ang-(1–7)	A-779	Ang-(1–7) + A-779
Body weight (g)	381 ± 11	385 ± 2	404 ± 18	399 ± 14
SBP (mm Hg)	136 ± 10	116 ± 7*	135 ± 7	132 ± 5
Glucose (mg/dl)	104 ± 7	108 ± 8	111 ± 4	109 ± 8
Insulin (ng/ml)	4.2 ± 0.8	1.3 ± 0.3*	5.2 ± 1.4	4.7 ± 0.9
Triglycerides (mg/dl)	84 ± 8	22 ± 9*	102 ± 17	84 ± 12
HOMA score	27.5 ± 5.8	8.2 ± 1.7*	35.6 ± 10.9	32.1 ± 5.7

Values are means ± SEM ($n = 6$ animals in each group). All groups of animals received a high fructose diet. Ang-(1–7): angiotensin-(1–7); SBP: systolic blood pressure. *Value significantly different from control saline value ($P < 0.05$).

3.5. Protocol 2: Angiotensin-(1–7) improves insulin-induced phosphorylation of Akt, GSK-3β and AS160 in skeletal muscle, adipose tissue and liver of FFR via a Mas receptor-dependent pathway

In agreement with our previous reports, chronic treatment with Ang-(1–7) improved the insulin-stimulated phosphorylation of Akt in skeletal muscle, adipose tissue and liver of FFR (Fig. 6). This change correlated with a significant improvement in the insulin-stimulated phosphorylation of GSK-3β in these tissues (Fig. 7 upper panel). In addition, FFR that received Ang-(1–7) exhibited a significant increase in the insulin-induced specific phosphorylation of AS160 in skeletal muscle and adipose tissue when compared to untreated FFR (Fig. 8, upper panel). To determine the participation of the Mas receptor in the beneficial effects exerted by Ang-(1–7) on mediators of insulin signal transduction, FFR were treated with a mixture of Ang-(1–7)

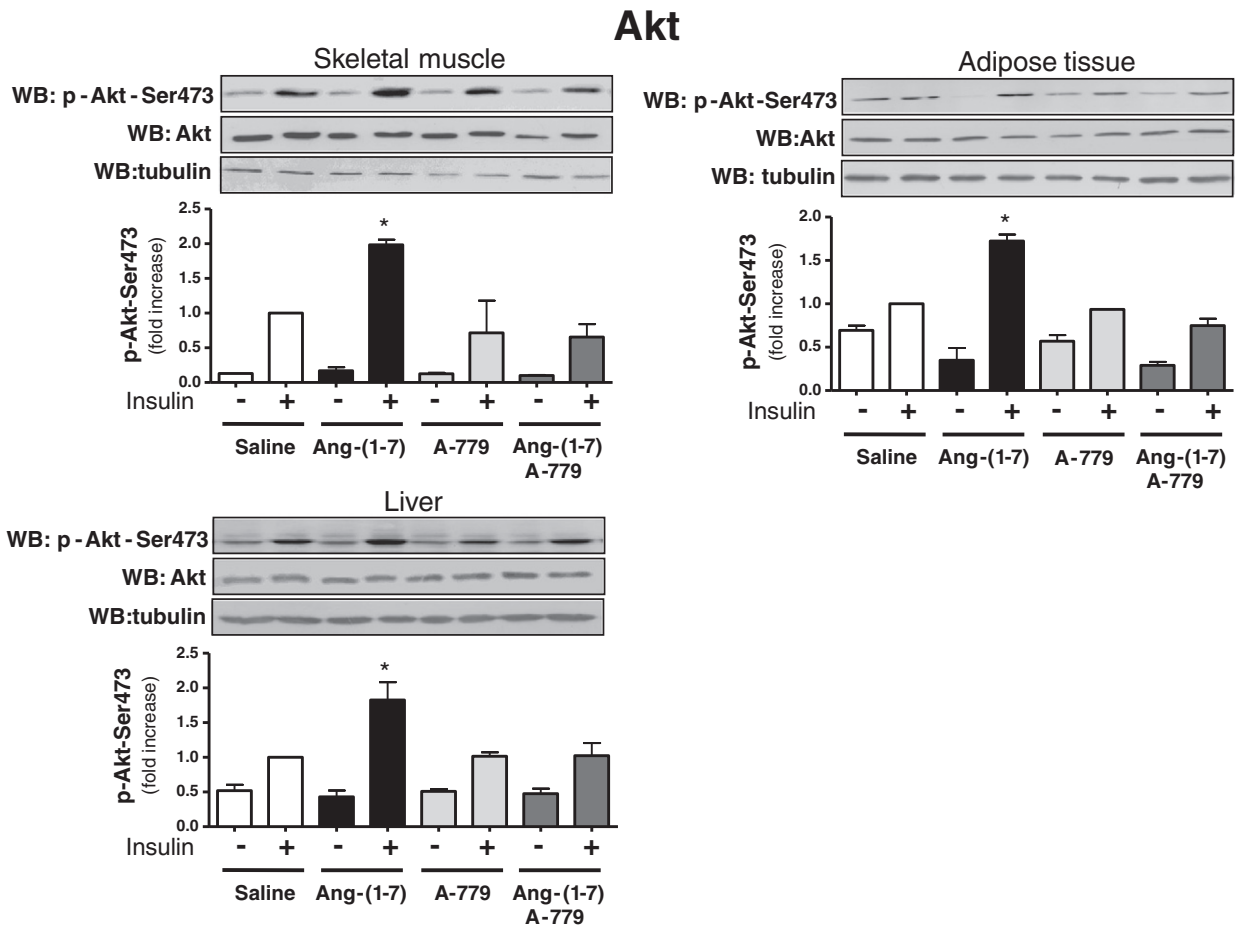

Fig. 6. Protocol 2: Akt-Ser473 phosphorylation in skeletal muscle, adipose tissue and liver of fructose-fed rats. After the two-week treatment with Ang-(1–7), A-779 or both compounds, rats were starved overnight, anesthetized were injected via portal vein with 10 IU of porcine insulin per kg body weight in normal saline (0.9% NaCl) in a final volume of 0.2 ml. To obtain data under basal conditions, rats received an injection of saline. The liver, adipose tissue (epididymal) and skeletal muscle (*soleus*) were removed after 1, 3 and 5 min respectively. Solubilized tissue proteins were subjected to immunoblotting with anti-phospho-Akt (p-Akt-Ser473) (upper panels). To determine protein abundance, total tissue extracts were subjected to western blotting with anti-Akt antibodies (lower panels). Bar graphs show the quantitative Akt-Ser473 phosphorylation. Data (means ± SEM) are expressed as fold increases in phosphorylation over basal ($n = 3$; $^*P < 0.05$). WB: western blotting.

and A-779 for 14 d and evaluated for the acute insulin response at Akt, GSK-3β and AS160 phosphorylation. As shown in Figs. 6 to 8, (upper panels), selective antagonism of the Mas receptor abolished the stimulating effects of Ang-(1–7) on Akt, GSK-3β and AS160 phosphorylation in all tissues analyzed. Administration of the Mas antagonist alone did not affect the phosphorylation of any signaling proteins analyzed (Figs. 6, 7 and 8, upper panels). The abundance of any of the analyzed protein abundance remained unchanged (Figs. 6 to 8, middle panels). Protein loading in gels was evaluated with an anti-tubulin antibody (lower panels).

4. Discussion

One of the major finding of the current study is that the beneficial effects exerted by Ang-(1–7) on crucial insulin signaling mediators *in vivo*, disappeared in the presence of an antagonist of the Ang-(1–7) specific Mas receptor. By acute administration of mixtures of hormones we determined that Ang II attenuates the insulin-stimulated phosphorylation of Akt, GSK-3β and AS160 in metabolic tissues (liver, adipose tissue and skeletal muscle). This result agrees with our previously reported observations made in rat heart *in vivo* as well as with *in vitro* studies employing aortic vascular smooth muscle cells [21,28]. By acute co-administration of insulin, Ang II, Ang-(1–7) alone or in combination we determined that Ang-(1–7) counteracts the inhibitory effect of Ang II on insulin-stimulated phosphorylation of Akt, GSK-3β and AS160. Through the use of A-779 we determined that these beneficial effects of Ang-(1–7) proceeded via a Mas receptor-dependent pathway. These results provide a further insight into the mechanisms involved in the positive effects on insulin action exerted by Ang-(1–7). Phosphorylation levels of Akt, GSK-3β and AS160 attained after the co-administration of insulin and Ang-(1–7) were comparable to those detected after acute stimulation with insulin alone, indicating that the ability of Ang-(1–7) to restore insulin-stimulated phosphorylation of Akt, GSK-3β and AS160 is not a consequence of an enhancement of the stimulating action of insulin, but the result of a counteraction of the negative acute modulation exerted by Ang II. This opposing action of Ang-(1–7) on the effects of Ang II has also been demonstrated for other Ang II-stimulated signaling pathway such as the mitogenic MAP kinase cascade, both in cells in culture [29–31] and in rat heart *in vivo* [32].

Additional novel information presented in the current work includes the study of mechanisms by which Ang-(1–7) treatment improves of glucose and lipid metabolism in insulin resistant FFR, an animal model that has many features in common with the human metabolic syndrome. There is little information about the regulation of Mas expression in FFR. We have previously reported that cardiac abundance of the Mas receptor is increased in FFR [33]. In the present study we report data from Mas expression in peripheral tissues of FFR. Interestingly, while Mas receptor abundance in liver and skeletal

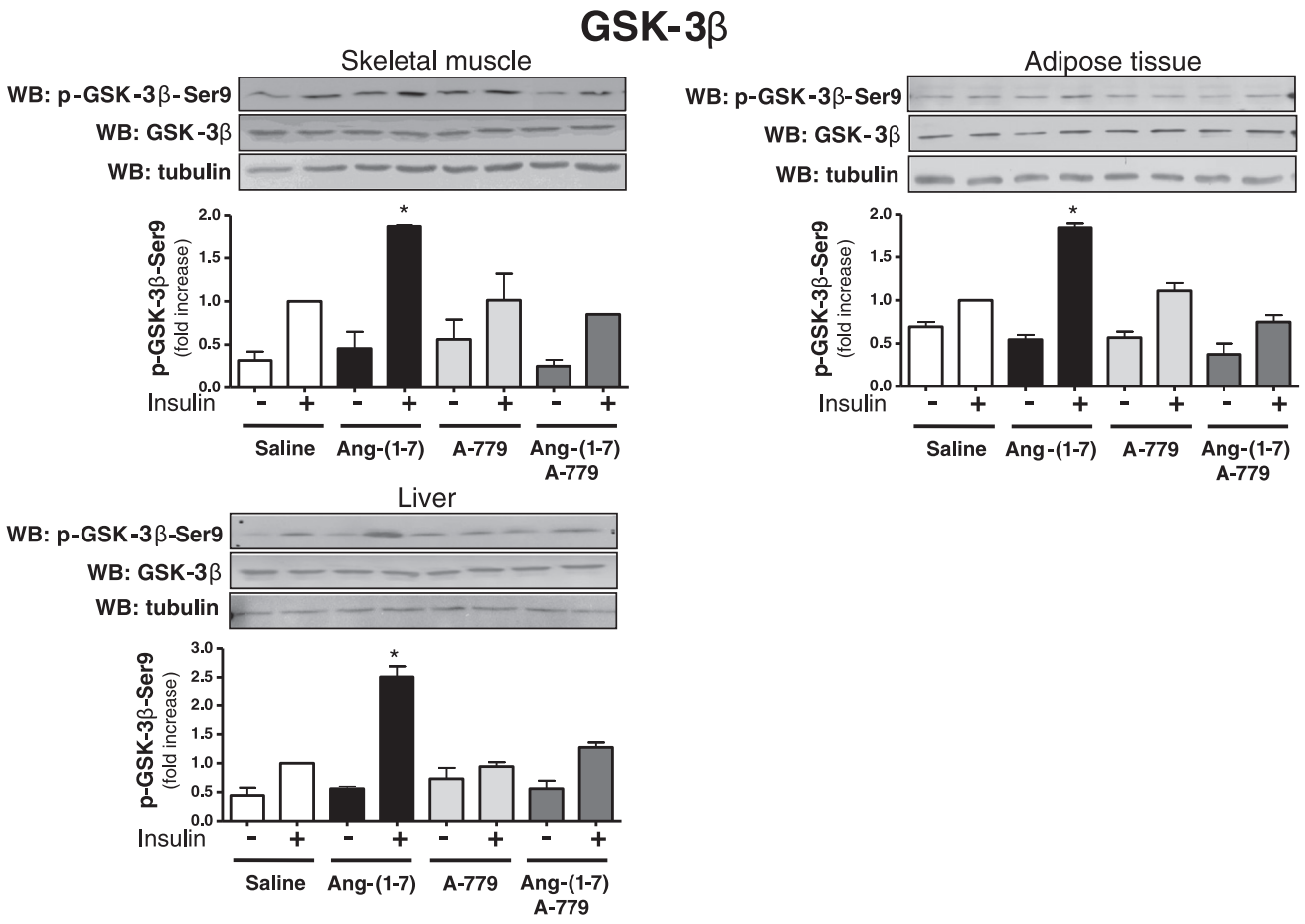

Fig. 7. Protocol 2: GSK-3β-Ser9 phosphorylation in skeletal muscle, adipose tissue and liver of fructose-fed rats. Rats were treated as described for Fig. 4. At the indicated time points, the liver, adipose tissue (epididymal) and skeletal muscle (soleus) were removed and homogenized as described in Methods. Solubilized tissue proteins were subjected to immunoblotting with anti-phospho-GSK-3β (p-GSK-3β-Ser9) (upper panels). To determine protein abundance, total tissue extracts were subjected to western blotting with anti-GSK-3β antibodies (lower panels). Bar graphs show the quantitative GSK-3β-Ser9 phosphorylation. Data (means ± SEM) are expressed as fold increases in phosphorylation over basal ($n = 3$; $^*P < 0.05$). WB: western blotting.

muscle remained unaltered after chronic treatment with Ang-(1–7), adipose tissue from FFR exhibited a significant increase in the protein abundance of the Mas receptor, suggesting an organ-specific regulation of local Mas expression by Ang-(1–7). Tissue-specific changes in the abundance of Mas receptor were also detected in a previous study performed in hypertensive SHR rats that were treated

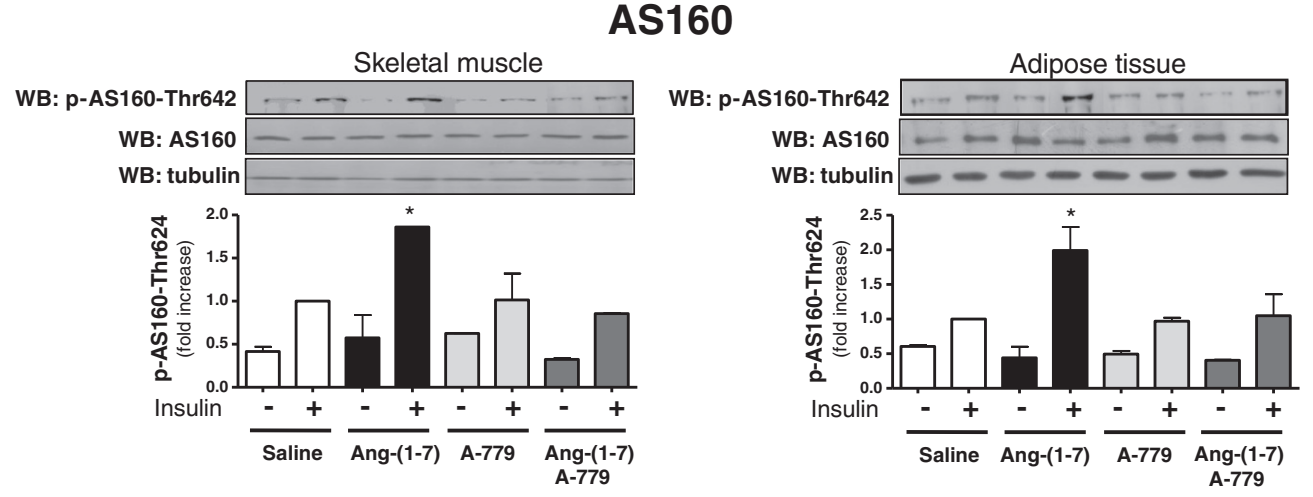

Fig. 8. Protocol 2: AS160-Thr642 phosphorylation in skeletal muscle and adipose tissue of fructose-fed rats. Rats were treated as described for Fig. 4. At the indicated time points, the liver, adipose tissue (epididymal) and skeletal muscle (soleus) were removed and homogenized as described in Methods. Solubilized tissue proteins were subjected to immunoblotting with anti-phospho-AS160 (p-AS160-Thr642) (upper panels). To determine protein abundance, total tissue extracts were subjected to western blotting with anti-AS160 antibodies (lower panels). Bar graphs show the quantitative AS160-Thr642 phosphorylation. Data (means ± SEM) are expressed as fold increases in phosphorylation over basal ($n = 3$; $^*P < 0.05$). WB: western blotting.

with Ang-(1–7) [34]. These regulatory effects of Ang-(1–7) on Mas receptor may influence the overall local RAS and participate in the progression and prognosis of diseases associated with metabolic syndrome.

In a previous study, we demonstrated that circulating Ang II levels are increased in FFR [20]. In addition, administration of both Ang II receptor antagonists [12,35–38] or ACE inhibitors [38] has been shown to revert the insulin resistance in FFR, strongly suggesting that Ang II is involved in the insulin resistant state developed by fructose overload. Accordingly, in the current work we showed that FFR displayed a marked immunostaining for Ang II in liver, skeletal muscle and adipose tissue. In view of this data, and given that Ang-(1–7) counteracts most of Ang II actions, we hypothesize that our current observation showing reversal of insulin resistance in FFR 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. These beneficial effects appeared to be mediated by a Mas receptor-dependent mechanism. In good agreement with our previous report [20], chronic Ang-(1–7) treatment resulted in a reversal of fructose-induced insulin resistance that included reduction of fasting triglyceride and insulin levels, reduction of systolic blood pressure and restoration of insulin response in terms of activity of Akt. This Ser/Thr kinase has been implicated as a key signaling protein for several insulin actions, including activation of glycogen synthesis, protein synthesis and GLUT-4 translocation to the cell surface, thereby increasing glucose transport [39]. One of the principal downstream targets of Akt is GSK-3β, a proline-directed Ser/Thr kinase that regulates a wide range of cellular processes including glycogen metabolism, gene transcription, protein translation, and cell apoptosis [39]. Under basal conditions, GSK-3β is highly active and inhibits glycogen synthesis by phosphorylation of glycogen synthase. Akt phosphorylates GSK-3β at inhibitory site Ser9, leading to stimulation of glycogen synthesis. Another important downstream target of Akt, is the so-called Akt substrate of 160 kDa (AS160), a Rab GTPase activating protein that regulates insulin-stimulated GLUT-4 trafficking [40]. Phosphorylation of AS160 by Akt is necessary for GLUT-4 translocation to occur [41]. In the current study we demonstrated that the Ang-(1–7)-induced enhancement of insulin action extends to two of the main downstream mediators of Akt: GSK-3β and AS160 in the main target tissues of insulin analyzed. We have previously demonstrated the presence of the Mas receptor in liver, adipose tissue and skeletal muscle through immunohistochemistry [22] suggesting the existence of a local ACE2/Ang-(1–7)/Mas receptor axis in these tissues. In the present study, we demonstrated the *in vivo* participation of the Mas receptor in the insulin-sensitizing effects of Ang-(1–7). When administered chronically in the presence of A-779, Ang-(1–7) failed, not only to revert insulin resistance in fructose-fed rats, but also to enhance the response of insulin in terms of Akt, GSK-3β and AS160 activation in liver, skeletal muscle and adipose tissue. Taken together, our findings agree with the demonstration that Ang-(1–7) stimulates the activation of Akt kinase via the Mas receptor in human endothelial cells [42]. Some of the effects reported for Ang-(1–7) have been shown to be mediated by AT1 and AT2 receptors [43]. However, in a previous work we have observed that antagonism of the AT1R with losartan or blockade of the AT2R with the selective antagonist PD123319 does not modify Ang-(1–7)-induced phosphorylation of Akt in rat heart [21], suggesting that these receptors are not involved in the current observations.

We have previously shown that acute administration of Ang-(1–7) stimulates the phosphorylation of Akt in rat heart, adipose tissue, skeletal muscle and liver *in vivo* through a Mas receptor/PI3K-dependent mechanism [21,22]. However, it is not determined whether Ang-(1–7) can counteract the deleterious actions of Ang II in the insulin signaling system *in vivo*. In this study, we proved that in contrast to the stimulating effects induced by Ang-(1–7), Ang II reduced the insulin-induced phosphorylation of Akt, as well as that of the downstream substrates GSK-3β and AS160, reinforcing our previously postulated notion that this enzyme could be a divergence signaling node in the transduction pathways of Ang II and Ang-(1–7) [21,22]. One potential mechanism involved in these beneficial effects of Ang-(1–7) could be the ability of Ang-(1–7) to activate, unlike Ang II, the enzyme Akt. A phenomenon that has been described both *in vitro* and *in vivo* [21,22,42]. It is important to consider that Ang-(1–7) antagonizes the inhibitory effects of Ang II on insulin-induced activation of Akt [21]. 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 FFR. Angiotensin-(1–7) is a vasodilator, in part due to its recently demonstrated capability of activating the endothelial nitric oxide synthase [42] and also because of its proven ability to potentiate the action of bradykinin [5]. The ability of Ang-(1–7) to activate proximal signaling downstream of the Mas receptor is poorly defined. A clear understanding of the relationship between Ang-(1–7) and Mas is complicated by its binding to and signaling through the angiotensin AT1 and AT2 receptors [43–46] and the potential formation of heterodimers between the Mas and AT1 receptors that may and alter the Mas receptor pharmacology [47,48]. Previous studies have shown that along with activation of endothelial nitric oxide synthetase and Akt, Ang-(1–7) causes internalization of the Mas receptor and induces the release of arachidonic acid, prostaglandins as well as release and potentiation of the actions of bradykinin [42,49–51]. However, Ang-(1–7) has not been shown to elicit changes in Ca^{2+} levels or stimulate inositol phosphate accumulation in tissue that express the Mas receptor (heart) or Mas over-expressing cells [52,53]. Moreover, Ang-(1–7) does not modulate Mas signaling through Gq, Gs or Gi. Thus, the sequence of events following Ang-(1–7) binding to Mas receptor remains unclear, and its protective effects appear to occur via a non-G-protein mechanism.

Given its positive effects on insulin action and signaling, Ang-(1–7) has a role in the modulation of glucose and lipid metabolism [20–22,24], the metabolic syndrome-like state displayed by Mas receptor knockout mice reinforces this novel role of Ang-(1–7). In the current study we provided further information on the mechanisms involved in these effects by analyzing the activation of downstream signaling components of Akt by Ang-(1–7) on the well characterized model of insulin resistance of fructose-fed, as well as the participation of the Mas receptor in the beneficial effects induced by Ang-(1–7). In accordance with previous reports, the favorable effects of chronic Ang-(1–7) treatment in insulin-resistant animals included reduction of fasting triglyceride and insulin levels, reduction of systolic blood pressure, and an improvement in the phosphorylation of Akt in the main target tissues of insulin: skeletal muscle, liver, and adipose tissue [20]. These effects extended to the activation of the downstream targets GSK-3β and AS160. In addition, we demonstrated that Mas receptor antagonism abolished the favorable effects of this hormone, since a two-week administration of A-779 reverted the stimulatory effect of Ang-(1–7) on metabolic parameters and insulin signaling components in the main target tissues analyzed.

In conclusion, our current findings suggest that Ang-(1–7) attenuates acute Ang II-mediated inhibition of insulin signaling components in normal rats via a Mas receptor-dependent mechanism. In addition, we found that the Mas receptor appears to be involved in the beneficial effects of Ang-(1–7) on crucial insulin signaling mediators (Akt, GSK-3β and AS160), in liver, muscle and adipose tissue of FFR. These results provide new insight into the mechanisms by which Ang-(1–7) exerts its positive physiological modulation of insulin actions in classical metabolic tissues and reinforces the central role of Akt in these effects.

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References

[1] Henriksen EJ. Improvement of insulin sensitivity by antagonism of the renin-angiotensin system. Am J Physiol Regul Integr Comp Physiol 2007;293:R974–80.
[2] Richey JM, Ader M, Moore D, Bergman RN. Angiotensin II induces insulin resistance independent of changes in interstitial insulin. Am J Physiol 1999;277:E920–6.
[3] Rao RH. Effects of angiotensin II on insulin sensitivity and fasting glucose metabolism in rats. Am J Hypertens 1994;7:655–60.
[4] Paul M, Poyan Mehr A, Kreutz R. Physiology of local renin-angiotensin systems. Physiol Rev 2006;86:747–803.
[5] Ferrario CM. New physiological concepts of the renin-angiotensin system from the investigation of precursors and products of angiotensin I metabolism. Hypertension 2010;55:445–52.
[6] Santos RA, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de Buhr I, Heringer-Walther S, Pinheiro SV, Lopes MT, Bader M, Mendes EP, Lemos VS, Campagnole-Santos MJ, Schultheiss HP, Speth R, Walther T. Angiotensin-(1–7) is an endogenous ligand for the G protein-coupled receptor Mas. Proc Natl Acad Sci U S A 2003;100:8258–63.
[7] Ferrario CM, Ahmad S, Joyner J, Varagic J. Advances in the renin angiotensin system focus on angiotensin-converting enzyme 2 and angiotensin-(1–7). Adv Pharmacol 2010;59:197–233.
[8] Velloso LA, Folli F, Perego L, Saad MJ. The multi-faceted cross-talk between the insulin and angiotensin II signaling systems. Diabetes Metab Res Rev 2006;22:98–107.
[9] Andraws R, Brown DL. Effect of inhibition of the renin-angiotensin system on development of type 2 diabetes mellitus (meta-analysis of randomized trials). Am J Cardiol 2007;99:1006–12.
[10] Jandeleit-Dahm KA, Tikellis C, Reid CM, Johnston CI, Cooper ME. Why blockade of the renin-angiotensin system reduces the incidence of new-onset diabetes. J Hypertens 2005;23:463–73.
[11] Henriksen EJ, Jacob S, Kinnick TR, Teachey MK, Krekler M. Selective angiotensin II receptor receptor antagonism reduces insulin resistance in obese Zucker rats. Hypertension 2001;38:884–90.
[12] Furuhashi M, Ura N, Takizawa H, Yoshida D, Moniwa N, Murakami H, Higashiura K, Shimamoto K. Blockade of the renin-angiotensin system decreases adipocyte size with improvement in insulin sensitivity. J Hypertens 2004;22:1977–82.
[13] Shiuchi T, Iwai M, Li HS, Wu L, Min LJ, Li JM, Okumura M, Cui TX, Horiuchi M. Angiotensin II type-1 receptor blocker valsartan enhances insulin sensitivity in skeletal muscles of diabetic mice. Hypertension 2004;43:1003–10.
[14] Muñoz MC, Argentino DP, Dominici FP, Turyn D, Toblli JE. Irbesartan restores the in-vivo insulin signaling pathway leading to Akt activation in obese Zucker rats. J Hypertens 2006;24:1607–17.
[15] Carvalho CR, Thirone AC, Gontijo JA, Velloso LA, Saad MJ. Effect of captopril, losartan, and bradykinin on early steps of insulin action. Diabetes 1997;46: 1950–7.
[16] Nawano M, Anai M, Funaki M, Kobayashi H, Kanda A, Fukushima Y, Inukai K, Ogihara T, Sakoda H, Onishi Y, Kikuchi M, Yazaki Y, Oka Y, Asano T. Imidapril, an angiotensin-converting enzyme inhibitor, improves insulin sensitivity by enhancing signal transduction via insulin receptor substrate proteins and improving vascular resistance in the Zucker fatty rat. Metabolism 1999;48:1248–55.
[17] Iyer SN, Ferrario CM, Chappell MC. Angiotensin-(1–7) contributes to the antihypertensive effects of blockade of the renin-angiotensin system. Hypertension 1998;31:356–61.
[18] Luque M, Martin P, Martell N, Fernandez C, Brosnihan KB, Ferrario CM. Effects of captopril related to increased levels of prostacyclin and angiotensin-(1–7) in essential hypertension. J Hypertens 1996;14:799–805.
[19] Stanziola L, Greene LJ, Santos RA. Effect of chronic angiotensin converting enzyme inhibition on angiotensin I and bradykinin metabolism in rats. Am J Hypertens 1999;12:1021–9.
[20] Giani JF, Mayer MA, Munoz MC, Silberman EA, Hocht C, Taira CA, Gironacci MM, Turyn D, Dominici FP. Chronic infusion of angiotensin-(1–7) improves insulin resistance and hypertension induced by a high-fructose diet in rats. Am J Physiol Endocrinol Metab 2009;296:E262–71.
[21] Giani JF, Gironacci MM, Munoz MC, Pena C, Turyn D, Dominici FP. Angiotensin-(1 7) stimulates the phosphorylation of JAK2, IRS-1 and Akt in rat heart *in vivo*: role of the AT1 and Mas receptors. Am J Physiol Heart Circ Physiol 2007;293:H1154–63.
[22] Muñoz MC, Giani JF, Dominici FP. Angiotensin-(1–7) stimulates the phosphorylation of Akt in rat extracardiac tissues *in vivo* via receptor Mas. Regul Pept 2010;161:1–7.
[23] Santos SH, Fernandes LR, Mario EG, Ferreira AV, Porto LC, Alvarez-Leite JI, Botion LM, Bader M, Alenina N, Santos RA. Mas deficiency in FVB/N mice produces marked changes in lipid and glycemic metabolism. Diabetes 2008;57:340–7.
[24] Santos SH, Braga JF, Mario EG, Porto LC, Rodrigues-Machado Mda G, Murari A, Botion LM, Alenina N, Bader M, Santos RA. Improved lipid and glucose metabolism in transgenic rats with increased circulating angiotensin-(1–7). Arterioscler Thromb Vasc Biol 2010;30:953–61.
[25] Bindom SM, Hans CP, Xia H, Boulares AH, Lazartigues E. Angiotensin I-converting enzyme type 2 (ACE2) gene therapy improves glycemic control in diabetic mice. Diabetes 2010;59:2540–8.
[26] Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. Anal Biochem 1985;150:76–85.
[27] Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28:412–9.
[28] Folli F, Saad MJ, Velloso L, Hansen H, Carandente O, Feener EP, Kahn CR. Crosstalk between insulin and angiotensin II signalling systems. Exp Clin Endocrinol Diabetes 1999;107:133–9.
[29] Ketsawatsomkron P, Stepp DW, Fulton DJ, Marrero MB. Molecular mechanism of angiotensin II-induced insulin resistance in aortic vascular smooth muscle cells: roles of Protein Tyrosine Phosphatase-1B. Vascul Pharmacol 2010;53:160–8.
[30] Su Z, Zimpelmann J, Burns KD. Angiotensin-(1–7) inhibits angiotensin II-stimulated phosphorylation of MAP kinases in proximal tubular cells. Kidney Int 2006;69:2212–8.
[31] Sampaio WO, Henrique de Castro C, Santos RA, Schiffrin EL, Touyz RM. Angiotensin-(1–7) counterregulates angiotensin II signaling in human endothelial cells. Hypertension 2007;50:1093–8.
[32] Giani JF, Gironacci MM, Munoz MC, Turyn D, Dominici FP. Angiotensin-(1–7) has a dual role on growth-promoting signalling pathways in rat heart *in vivo* by stimulating STAT3 and STAT5a/b phosphorylation and inhibiting angiotensin II-stimulated ERK1/2 and Rho kinase activity. Exp Physiol 2008;93:570–8.
[33] Giani JF, Munoz MC, Mayer MA, Veiras LC, Arranz C, Taira CA, Turyn D, Toblli JE, Dominici FP. Angiotensin-(1–7) improves cardiac remodeling and inhibits growth-promoting pathways in the heart of fructose-fed rats. Am J Physiol Heart Circ Physiol 2010;298:H1003–13.
[34] Tan Z, Wu J, Ma H. Regulation of angiotensin-converting enzyme 2 and Mas receptor by Ang-(1–7) in heart and kidney of spontaneously hypertensive rats. J Renin Angiotensin Aldosterone Syst 2011;12:413–9.
[35] Kamari Y, Harari A, Shaish A, Peleg E, Sharabi Y, Harats D, Grossman E. Effect of telmisartan, angiotensin II receptor antagonist, on metabolic profile in fructose-induced hypertensive, hyperinsulinemic, hyperlipidemic rats. Hypertens Res 2008;31:135–40.
[36] Hsieh PS. Reversal of fructose-induced hypertension and insulin resistance by chronic losartan treatment is independent of AT2 receptor activation in rats. J Hypertens 2005;23:2209–17.
[37] Okada K, Hirano T, Ran J, Adachi M. Olmesartan medoxomil, an angiotensin II receptor blocker ameliorates insulin resistance and decreases triglyceride production in fructose-fed rats. Hypertens Res 2004;27:293–9.
[38] Higashiura K, Ura N, Takada T, Li Y, Torii T, Togashi N, Takada M, Takizawa H, Shimamoto K. The effects of an angiotensin-converting enzyme inhibitor and an angiotensin II receptor antagonist on insulin resistance in fructose-fed rats. Am J Hypertens 2000;13:290–7.
[39] Taniguchi CM, Emanuelli B, Kahn CR. Critical nodes in signalling pathways: insights into insulin action. Nat Rev Mol Cell Biol 2006;7:85–96.
[40] Kane S, Sano H, Liu SC, Asara JM, Lane WS, Garner CC, Lienhard GE. A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain. J Biol Chem 2002;277:22115–8.
[41] Sano H, Kane S, Sano E, Miinea CP, Asara JM, Lane WS, Garner CW, Lienhard GE. Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. J Biol Chem 2003;278:14599–602.
[42] Sampaio WO, Souza dos Santos RA, Faria-Silva R, da Mata Machado LT, Schiffrin EL, Touyz RM. Angiotensin-(1–7) through receptor Mas mediates endothelial nitric oxide synthase activation via Akt-dependent pathways. Hypertension 2007;49: 185–92.
[43] Walters PE, Gaspari TA, Widdop RE. Angiotensin-(1–7) acts as a vasodepressor agent via angiotensin II type 2 receptors in conscious rats. Hypertension 2005;45:960–6.
[44] Gironacci MM, Coba MP, Pena C. Angiotensin-(1–7) binds at the type 1 angiotensin II receptors in rat renal cortex. Regul Pept 1999;84:51–4.
[45] De Souza AM, Lopes AG, Pizzino CP, Fossari RN, Miguel NC, Cardozo FP, Abi-Abib R, Fernandes MS, Santos DP, Caruso-Neves C. Angiotensin II and angiotensin-(1–7) inhibit the inner cortex Na+-ATPase activity through AT2 receptor. Regul Pept 2004;120:167–75.
[46] Kostenis E, Milligan G, Christopoulos A, Sanchez-Ferrer CF, Heringer-Walther S, Sexton PM, Gembardt F, Kellett E, Martini L, Vanderheyden P, Schultheiss HP, Walther T. G-protein-coupled receptor Mas is a physiological antagonist of the angiotensin II type 1 receptor. Circulation 2005;111:1806–13.
[47] Bosnyak S, Jones ES, Christopoulos A, Aguilar MI, Thomas WG, Widdop RE. Relative affinity of angiotensin peptides and novel ligands at AT1 and AT2 receptors. Clin Sci (Lond) 2011;121:297–303.
[48] Lyngso C, Erikstrup N, Hansen JL. Functional interactions between 7TM receptors in the renin-angiotensin system–dimerization or crosstalk? Mol Cell Endocrinol 2009;302:203–12.
[49] Gironacci MM, Adamo HP, Corradi G, Santos RA, Ortiz P, Carretero OA. Angiotensin (1–7) induces MAS receptor internalization. Hypertension 2011;58:176–81.
Trachte GJ, Meixner K, Ferrario CM, Khosla MC. Prostaglandin production in response to angiotensin-(1–7) in rabbit isolated vasa deferentia. Prostaglandins 2011;39: 385–94.
[50] Porsti I, Bara AT, Busse R, Hecker M. Release of nitric oxide by angiotensin-(1–7) from porcine coronary endothelium: implications for a novel angiotensin receptor. Br J Pharmacol 1994;111:652–4.
[51] Muthalif MM, Benter IF, Uddin MR, Harper JL, Malik KU. Signal transduction mechanisms involved in angiotensin-(1–7)-stimulated arachidonic acid release and prostanoid synthesis in rabbit aortic smooth muscle cells. J Pharmacol Exp Ther 1998;284:388–98.
[52] Dias-Peixoto MF, Santos RA, Gomes ER, Alves MN, Almeida PW, Greco L, Rosa M, Fauler B, Bader M, Alenina N, Guatimosim S. Molecular mechanisms involved in the angiotensin-(1–7)/Mas signaling pathway in cardiomyocytes. Hypertension 2008;52:542–8.
[53] Shemesh R, Toporik A, Levine Z, Hecht I, Rotman G, Wool A, Dahary D, Gofer E, Kliger Y, Soffer MA, Rosenberg A, Eshel D, Cohen Y. Discovery and validation of novel peptide agonists for G-protein-coupled receptors. J Biol Chem 2008;283: 34643–9.