Regulatory Peptides 161 (2010) 1–7

Contents lists available at ScienceDirect

Regulatory Peptides

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

Rapid communication

Angiotensin-(1-7) stimulates the phosphorylation of Akt in rat extracardiac tissues *in vivo* via receptor Mas

Marina C. Muñoz, Jorge F. Giani, Fernando P. Dominici *

Instituto de Química y Fisicoquímica Biológicas (IQUIFIB), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 7 September 2009
Received in revised form 5 January 2010
Accepted 11 February 2010
Available online 25 February 2010

Keywords:
Angiotensin II
Angiotensin-(1-7)
Akt
GSK-3ß
Mas receptor

ABSTRACT

The *in vivo* effect of angiotensin (ANG)-(1-7) on the activation of insulin signaling transduction in rat extracardiac tissues is unknown. Thus, in the present study, we evaluated the ability of ANG-(1-7) to stimulate the phosphorylation of Akt, a main mediator of insulin action in rat extracardiac tissues (adipose tissue, liver and skeletal muscle). We proved that ANG-(1-7) induces the phosphorylation of Akt at both threonine 308 and serine 473 in all tissues analyzed. Selective antagonism of the Mas receptor with A779 blocked the ANG-(1-7)-induced Akt phosphorylation in extracardiac tissues. Reinforcing this evidence, we determined that ANG-(1-7) induces the *in vivo* activation of the downstream target of Akt, glycogen synthase kinase-3β in liver and skeletal muscle. Moreover, in every tissue analyzed, the presence of the Mas receptor was detected by immunohistochemical analysis. Based on the current results, we postulate that ANG-(1-7) could be a positive physiological contributor to the actions of insulin in extracardiac tissues. Therefore, our findings extend the possibilities for new approaches in the study of ANG-(1-7)/Mas receptor axis and show the therapeutic potential of ANG-(1-7) in the treatment of metabolic disorders such as insulin resistance as well as other disorders associated with diminished Akt activity.

1. Introduction

Alterations within the renin–angiotensin system (RAS) are important contributors to the development of insulin resistance [1–3]. At least two opposing arms can be identified within the current view of the RAS. The first arm is represented by angiotensin II (ANG II) through its specific AT1 receptor (AT1R), and is characterized by hypertensive, proliferative and hypertrophic effects. ANG II is originated from an enzymatic cascade in which angiotensinogen is converted to ANG I and then to ANG II by the actions of renin and angiotensin converting enzyme (ACE), respectively [4,5]. The second arm is antihypertensive and antihypertrophic of nature, where the major participant is ANG-(1-7), an heptapeptide that constitutes an important functional end-product of the RAS and is primarily formed from ANG II by angiotensin converting enzyme type 2 (ACE2) [4,5]. The heptapeptide ANG-(1-7), through its specific G protein-coupled receptor Mas, induces responses opposing those of ANG II, including antihypertensive, antihypertrophic, antifibrotic, and antithrombotic properties [4–6].

A large body of evidence indicates that ANG II plays a critical role in the etiology of insulin resistance [7]. The mechanism behind this deleterious effect appears to be related to a negative modulation exerted by ANG II on several steps of the insulin signaling cascade, including insulin-induced phosphorylation of the insulin receptor (IR), insulin receptor substrate-1 (IRS-1) and activation of Akt by phosphatidylinositol 3-kinase (PI3K) [7]. This evidence clearly indicates that the signaling cross-talk between insulin and ANG II has significant physiological relevance.

In a previous work, we demonstrated that ANG-(1-7) is able to utilize components of the insulin signaling pathway by inducing IRS-1 and Akt phosphorylation via Mas receptor in rat heart *in vivo* [8]. In addition, it was shown that ANG-(1-7) overcomes the inhibition of insulin-induced phosphorylation of Akt phosphorylation by ANG II [8]. Our results are in keeping with studies performed in rat ventricular cardiomyocytes, confirming the involvement of the PI3K/Akt pathway in the mechanism of action of ANG-(1-7) [9]. Overall, these actions of ANG-(1-7) on insulin pathways indicated a role in metabolic processes.

In an effort to characterize this novel action of ANG-(1-7), Santos et al. analyzed the phenotype of Mas receptor-knockout mice reporting that genetic deletion of Mas receptor leads to a metabolic syndrome state in mice [10]. In a recent work, we confirmed the role of ANG-(1-7) as a modulator of insulin action [11]. Chronic infusion of the heptapeptide reverted insulin resistance by enhancing insulin signaling in insulin target tissues of fructose-fed rats [11]. We hypothesized that this insulin-sensitizing effect of ANG-(1-7) could be related to its capability of modulating Akt activity in other tissues aside the heart. Thus, in the present study, we evaluated the ability of ANG-(1-7) to stimulate the phosphorylation of Akt *in vivo* in the main insulin target tissues (adipose tissue, liver and skeletal muscle). Results were compared to those obtained with ANG II under the same

* Corresponding author. Instituto de Química y Fisicoquímica Biológicas (UBA-CONICET), Facultad de Farmacia y Bioquímica, Junín 956, 1113 Buenos Aires, Argentina. Tel.: +54 11 4964 8290x114; fax: +54 11 4962 5457.
E-mail address: dominici@qb.ffyb.uba.ar (F.P. Dominici).

0167-0115/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.regpep.2010.02.001

conditions. By the use of the selective receptor antagonist A779, we analyzed the participation of the Mas receptor in these signaling events activated by ANG-(1-7).

2. Materials and methods

2.1. Reagents

The peptides ANG II, ANG-(1-7) and [^{7}-D-Ala]-ANG-(1-7), (A779) were synthesized in our laboratory as described previously [8]. The reagents and apparatus for SDS-PAGE and immunoblotting were obtained from Bio-Rad (Hercules, CA, USA). The phospho-Akt (Thr308) rabbit polyclonal antibody that detects endogenous levels of Akt only when phosphorylated at threonine 308 (9275), the phospho-Akt (Ser473) rabbit polyclonal antibody that detects endogenous levels of Akt only when phosphorylated at serine 473 (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 β only when phosphorylated at serine 9 (9336), and the monoclonal antibody that detects total levels of GSK-3β (9315) were purchased from Cell Signaling (Beverly, MA, USA). 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 from Amersham (Piscataway, NJ, USA). The polyclonal anti-Mas Proto-Oncogene (anti-Mas receptor) antibody was purchased from Novus Biologicals (Littleton, CO, USA). The remaining reagents, including the polyclonal anti-β actin antibody were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Animals

Male Sprague–Dawley rats at 8 weeks of age were used. 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, hormones administration and tissue preparation

The experimental procedure was performed following previously described protocols [8,11]. 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) or ANG II (8 pmol/kg) or ANG-(1-7) (8 pmol/kg) into the portal vein. For selective antagonism of the Mas receptor, A779 (80 pmol/kg) was administered intravenously with or without ANG-(1-7) (8 pmol/kg).

In a previous work, we have 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 [8]. A similar level of phosphorylation was found within 10 min of stimulation [8]. Considering these results we decide to perform the experiment within 5–10 min after the stimulation with solutions containing different compositions [ANG-(1-7) or ANG II or normal saline or A779 or a mixture of A779 + ANG-(1-7)] 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 liver, which peaked 1–2 min after injection of an 8 pmol/kg dose of the hormone. According to this, the liver, the adipose tissue (epididymal) and the skeletal muscle (soleus) were removed after 1, 3 and 5 min, respectively, and kept at 80 °C until analysis or fixed in 10% buffered formaldehyde and embedded in paraffin.

Tissue samples were homogenized in 10 volumes of a solubilization buffer containing 1% Triton together with phosphates and protease inhibitors as described previously [11]. 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 measured using the Bradford method as described previously [11].

2.4. Immunoblotting

To determine the phosphorylation levels of Akt (Thr308 and Ser473), equal amounts of solubilized proteins (40 µg) were denatured by being boiled in reducing sample buffer, resolved by SDS-PAGE, and subjected to immunoblotting with the anti-phospho-Akt-Thr308 antibody (1:5000 dilution) or the anti-phospho-Akt-Ser473 antibody (1:3000 dilution). Akt abundance was detected by incubation of the membranes with the anti-Akt antibody (1:1000 dilution). The phosphorylation levels of GSK-3β were determined by subjecting tissue homogenates to Western Blotting using the anti-phospho-GSK-3β-Ser9 antibody (1:1000 dilution). Tissular abundance of GSK-3β was measured by immunoblotting using the anti-GSK-3β antibody (1:3000 dilution).

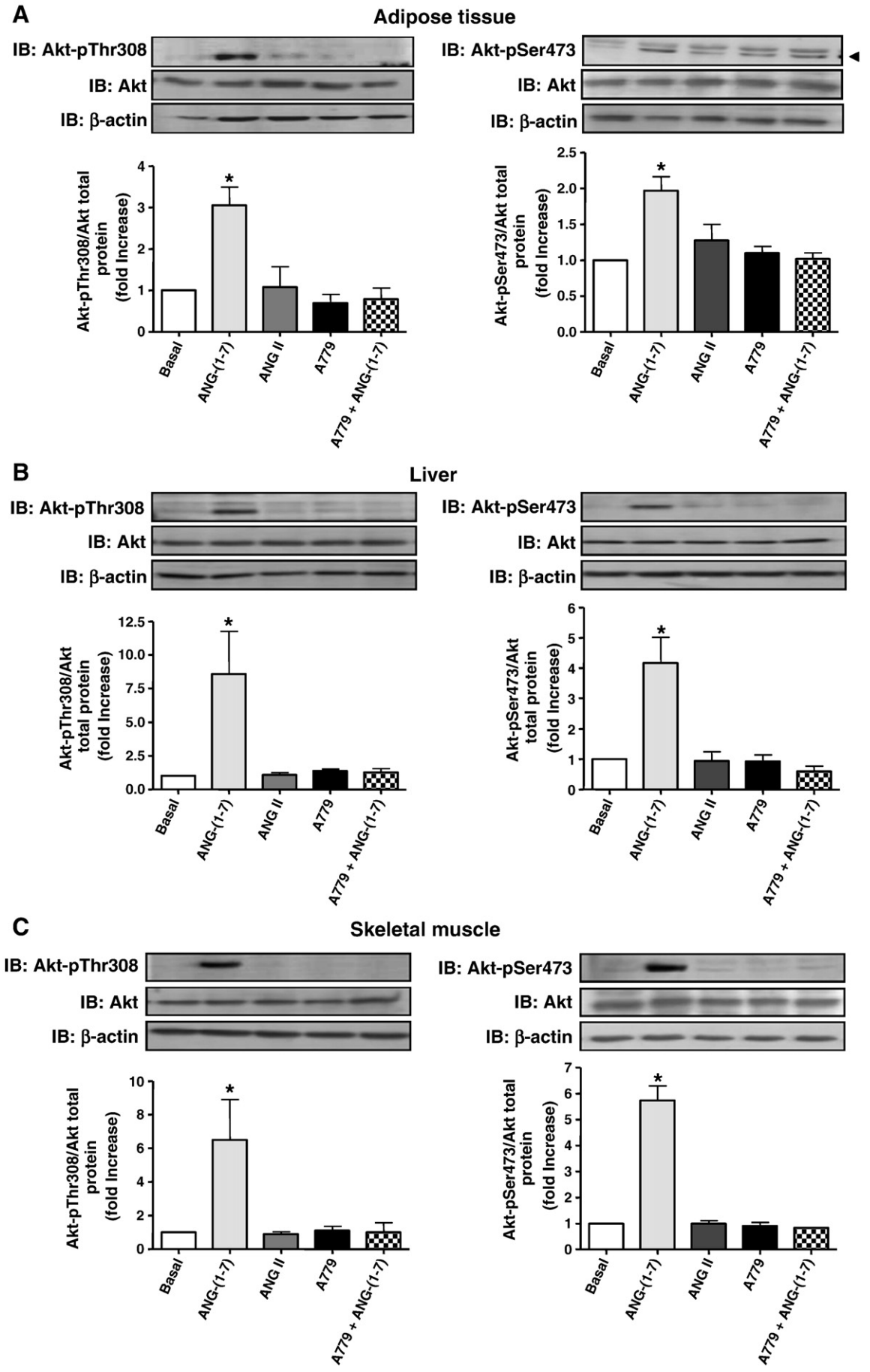
Finally, membranes were incubated for 1 h with goat anti-rabbit IgG-HRP secondary antibody, proteins detected by ECL and the intensities of the specific bands were quantitated by optical densitometry.

2.5. Immunohistochemical staining

Paraffin tissue sections (sagittal) 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. Mas receptor was detected using the anti-Mas receptor antibody (Novus Biologicals, Littleton, CO, USA) at a 1:100 dilution using a previously described protocol [12]. Sections were treated with biotinylated horse anti-rabbit/mouse/goat IgG (1:200 dilution) for 30 min at room temperature and incubated with the avidin–biotin–peroxidase complex (Vector, Burlingame, CA, USA) for 60 min. Peroxidase activity was visualized by exposing the sections for 1 min to 3,30-diaminobenzidine tetrahydrochloride (Vector, Burlingame, CA, USA) 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 Mas receptor immunostaining

Fig.1. ANG-(1-7)-induces Akt phosphorylation at residues Thr308 and Ser473 in rat adipose tissue, liver and skeletal muscle *in vivo*. Rats were anesthetized and acutely treated with a single i.v dose (0.2 ml) of a solution of normal saline or solutions of normal saline containing ANG II (8 pmol/kg), ANG-(1-7) (8 pmol/kg), A779 (80 pmol/kg) or a mixture of ANG-(1-7) (8 pmol/kg) and A779 (80 pmol/kg). Adipose tissue (A), liver (B), skeletal muscle (soleus; C) were removed and homogenized as described in Materials and methods (Section 2.3). Solubilized tissues proteins were subjected to immunoblotting (IB) with anti-phospho-Akt (Akt-pThr308 or Akt-pSer473) (upper panels). To determine Akt protein abundance, the same extracts were subjected to Western Blotting with anti-Akt (middle panels). Protein loading in gels was evaluated by reblotting membranes with anti-β-actin antibody (lower panels). Data (means ± S.E.M) are expressed as fold increases in phosphorylation over basal, ($n = 3$; $^*P < 0.01$ vs. basal value). Phosphorylation/protein ratio was corrected by actin content.


A
Adipose tissue
IB: Akt-pThr308
IB: Akt
IB: β-actin
IB: Akt-pSer473
IB: Akt
IB: β-actin
Akt-pThr308/Akt total protein (fold Increase)
Akt-pSer473/Akt total protein (fold Increase)
Basal
ANG-(1-7)
ANG II
A779
A779 + ANG-(1-7)
B
Liver
IB: Akt-pThr308
IB: Akt
IB: β-actin
IB: Akt-pSer473
IB: Akt
IB: β-actin
Akt-pThr308/Akt total protein (fold Increase)
Akt-pSer473/Akt total protein (fold Increase)
Basal
ANG-(1-7)
ANG II
A779
A779 + ANG-(1-7)
C
Skeletal muscle
IB: Akt-pThr308
IB: Akt
IB: β-actin
IB: Akt-pSer473
IB: Akt
IB: β-actin
Akt-pThr308/Akt total protein (fold Increase)
Akt-pSer473/Akt total protein (fold Increase)
Basal
ANG-(1-7)
ANG II
A779
A779 + ANG-(1-7)

presence in all tissues analyzed. All histological sections were studied in each animal using a light microscope (Olympus BX-51). Images were acquired with QCapture Pro software (QImaging, Surrey, BCCanada).

2.6. Statistical analysis

Data were analyzed with 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). A value of $P<0.05$ was considered significant. All values are presented as means ± S.E.M.

3. Results

3.1. ANG-(1-7) induces the phosphorylation of Akt in rat adipose tissue, liver, and skeletal muscle in vivo through a Mas receptor-dependent mechanism

To investigate if ANG-(1-7) induces the activation of Akt, we evaluated the effect of an acute *in vivo* administration of ANG-(1-7) on Akt phosphorylation in adipose tissue, liver, and skeletal muscle. As shown in Fig. 1, ANG-(1-7) induced the phosphorylation of Akt at residues Thr308 and Ser473 after the injection of an 8 pmol/kg dose of the hormone (upper panels, $n=3$ for all tissues). Under the same conditions, ANG II was unable to induce the phosphorylation of Akt in any of the analyzed tissues (Fig. 1, A–C). Total Akt protein was analyzed in each case by subjecting the corresponding membranes to immunoblotting with anti-Akt antibody (Fig. 1, middle panels). Protein loading in gels was evaluated and corrected by reblotting membranes with anti-β-actin antibody (Fig. 1, lower panels).

As shown in Fig. 1 (A–C, upper panels), the Mas receptor antagonist, A779 blocked the stimulating effects of ANG-(1-7) on Akt Thr308 and Ser473 phosphorylation ($n=3$ for all tissues). As expected, the administration of the Mas antagonist by itself did not affect the phosphorylation of Akt in any of the tissues studied (Fig. 1). Total amount of Akt protein analyzed was not affected by different treatments as confirmed by submitting the corresponding tissues extracts to immunoblotting with anti-Akt (Fig. 1, middle panels). Protein loading in the gels was evaluated and corrected after subjecting the corresponding membranes to reblotting with an anti-β-actin antibody (Fig. 1, lower panels).

3.2. ANG-(1-7) induces the phosphorylation of GSK-3β in rat liver and skeletal muscle in vivo through a Mas receptor-dependent mechanism

As shown in Fig. 2, after the intravenous injection of an 8 pmol/kg dose of ANG-(1-7), the phosphorylation of GSK-3β at Ser9 increased markedly in rat liver and skeletal muscle in comparison to basal values (Fig. 2, A, B, upper panels, $n=3$ for both tissues). Under the same conditions, ANG II did not induce the phosphorylation of GSK-3β (Fig. 2, A, B, upper panels).

As shown in Fig. 2 (A, B, upper panels), the Mas receptor antagonist A779 blocked the stimulating effects of ANG-(1-7) on GSK-3β Ser9 phosphorylation ($n=3$ for both tissues). The administration of the Mas antagonist by itself did not affect the phosphorylation of GSK-3β Ser9 phosphorylation (Fig. 2, A, B, upper panels).

As confirmed by immunoblotting with anti-GSK-3β, no changes in the abundance of GSK-3β in liver and skeletal muscle were detected after the different treatments (Fig. 2, A, B, middle panels). Protein loading in the gels was evaluated and corrected by reblotting the corresponding membranes with anti-β-actin antibody (Fig. 2, A, B, lower panels).

3.3. Mas receptor levels in extracardiac tissues of experimental animals

As shown in Fig. 3 (A–C), Mas receptor protein immunoreactivity was detected in sections from adipose tissue, liver and skeletal muscle.

4. Discussion

The role of the RAS extends well beyond its classical role in blood pressure regulation and salt-water balance. Local RAS have been described in a number of tissues, including cardiovascular, brain, endocrine, sensory, fat and immune cells; given its function in physiological and pathophysiological processes, such as metabolic diseases offers new therapeutic opportunities for pharmacological manipulation of this system [13].

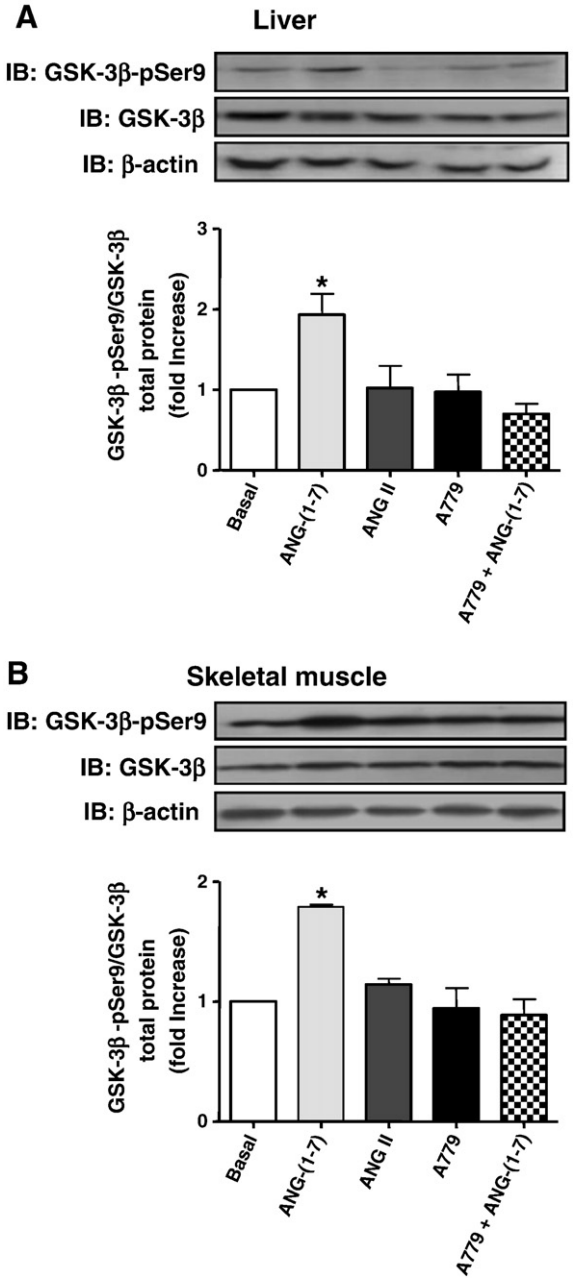


Fig.2. ANG-(1-7)-induces GSK-3β phosphorylation at Ser9 in rat liver and skeletal muscle *in vivo*. Rats were injected as described in Materials and methods (Section 2.3). At the indicated time points (see Section 2.3), liver (A) and skeletal muscle (soleus; B) were removed and homogenized as described in Materials and methods. Solubilized tissues proteins were subjected to immunoblotting (IB) with anti-phospho-GSK-3β (GSK-3β-pSer9) (upper panels). To determine GSK-3β protein abundance, the same extracts were subjected to Western Blotting with anti-GSK-3β (middle panels). Protein loading in gels was evaluated by reblotting membranes with anti-β-actin antibody (lower panels). Data (means ± S.E.M) are expressed as fold increases in phosphorylation over basal, ($n=3$; *$P<0.01$ vs. basal value). Phosphorylation/protein ratio was corrected by actin content.

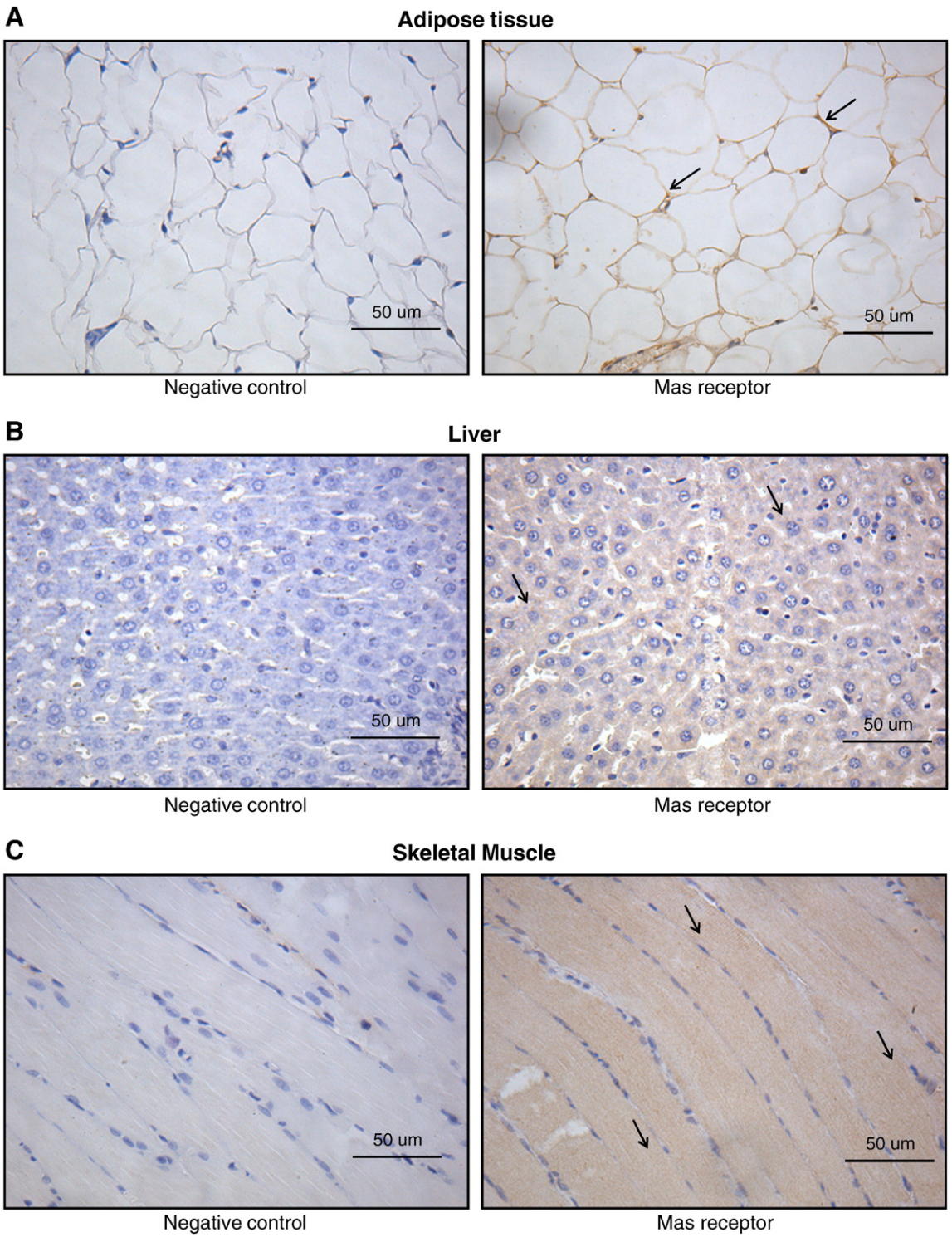


Fig.3. Representative tissue sections showing positive immunostaining for Mas receptor in rat adipose tissue (A), liver (B) and skeletal muscle (soleus; C). Original magnification × 400. All tissue sections show extended areas of positive staining for Mas receptor (indicated by arrows).

Angiotensin II, one of the most important active peptides of the RAS, plays a critical role in the etiology of insulin resistance [1–3]. The mechanism behind this deleterious effect appears to be related to a negative modulation exerted by ANG II on several steps of the insulin signaling cascade, including insulin-induced phosphorylation of the IR, IRS-1 and activation of Akt by phosphatidylinositol 3-kinase [7]. Accordingly, selective blockade of the AT1R or inhibition of ACE improves insulin sensitivity [3,7,14–16]. Considering that inhibition of ACE or chronic blockade of AT1Rs is associated with increased levels of circulating ANG-(1-7), this hormone could be involved in the beneficial effects of antihypertensive therapy [4,17–19]. Interestingly, acute blockade of ANG-(1-7) synthesis reverses some beneficial effects associated with RAS inhibition [18]. The counterregulatory effects of ANG-(1-7) on the pressor and trophic actions of ANG II appear to be mediated by the Mas receptor, a G protein-coupled receptor present in several tissues, including heart and kidney [19,20].

Activation of PI3K has been demonstrated to be a pivotal event in the metabolic actions of insulin [21]. An important downstream target

of the 3-phosphoinositides generated by PI3K is the Ser/Thr kinase Akt, which appears as a critical mediator of many insulin actions [21]. Akt is activated by phosphorylation at two regulatory sites, Thr308 and Ser473 [21]. In good concordance with our present results, through the use of the selective Mas receptor antagonist A779 and the specific PI3K inhibitor wortmanin, we have previously shown that acute administration of ANG-(1-7) stimulates the rapid phosphorylation of Akt in rat heart *in vivo* through a Mas receptor/PI3K-dependent mechanism [8]. However the *in vivo* effect of ANG-(1-7) on the activation of Akt in rat extracardiac tissues is unknown. Thus, the major goal of the current study was to determine if ANG-(1-7) is able to induce the phosphorylation of Akt in liver, adipose tissue and skeletal muscle *in vivo*.

We proved that ANG-(1-7) induces the *in vivo* phosphorylation of Akt at Thr308 and Ser473 in all rat tissues analyzed. In accordance with our previous results in rat heart tissue [8], we also demonstrated that under the same conditions, ANG II does not stimulate Akt phosphorylation in these tissues, reinforcing the previously postulated notion that this enzyme could be a divergence signaling node in the transduction pathways of ANG II and ANG-(1-7) [8].

Notably, in the current study we have also established that selective antagonism of the Mas receptor completely blocked the ANG-(1-7)-induced phosphorylation of Akt. This observation suggests the participation of the Mas receptor in the stimulation of Akt by ANG-(1-7). In support of this evidence, the presence of the Mas receptor was detected in all tissues analyzed through immunohistochemistry. This result is in good agreement with a previously reported study showing low levels of mRNA expression of the *mas* proto-oncogene in liver and skeletal muscle [22]. Taken together, our findings agree with those recently reported by Sampaio et al., which demonstrated that in human endothelial cells, ANG-(1-7), through receptor Mas, stimulates the activation of Akt kinase [23].

Although some of the effects reported for ANG-(1-7) have been shown to be mediated by the ANG II receptors AT1 and AT2 [6], in a previous work we have shown that antagonism of the AT1R with losartan or blockade of the AT2R with the selective antagonist PD123319 does not alter ANG-(1-7)-induced phosphorylation of Akt in rat heart suggesting that none of these receptors are involved in this response [8]. However, we have demonstrated that acute ANG-(1-7) administration leads to phosphorylation of other signaling molecules in the heart such as JAK2 and IRS-1 [8]. Unlike what was observed for Akt, involvement of the AT1R in this phenomenon was demonstrated by antagonism with losartan [8]. In good concordance with our present results, through the use of the selective Mas receptor antagonist A779 and the specific PI3K inhibitor wortmanin, we have previously shown that acute administration of ANG-(1-7) stimulates the rapid phosphorylation of cardiac Akt *in vivo* through a Mas receptor/PI3K-dependent mechanism [8].

Recently, Santos et al. [10] reported that genetic deletion of the Mas receptor leads to a metabolic syndrome state in mice, supporting a putative new physiological function of ANG-(1-7) in glycemic control and insulin resistance. Accordingly, in a recent work, we have demonstrated that chronic treatment with ANG-(1-7) results in a reversal of fructose-induced insulin resistance [11]. 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 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 [11]. These findings indicate that the ANG-(1-7)/Mas receptor axis is involved in metabolic processes and provide new insights into possible insulin sensitization mechanisms of ANG-(1-7).

To our knowledge, this is the first study to characterize ANG-(1-7) signal transduction in rat extracardiac tissues *in vivo*. Our current results indicate that Akt and GSK-3β could be possible physiological mediators of the insulin-sensitizing actions of ANG-(1-7). These results are in good concordance with our previous findings showing that chronic ANG-(1-7) treatment can revert insulin resistance an improve insulin signaling in fructose-fed rats [11]. Particularly, the finding that intravenous administration of ANG-(1-7) induces the activation of GSK-3β, a key regulator in glycogen synthesis and therefore in glucose homeostasis [24], points to ANG-(1-7) as a positive modulator of insulin action in liver and skeletal muscle.

In the last years, it has been demonstrated that ACE2 gene and tissue activity are up-regulated by chronic liver injury in human and bile duct ligated rat. Also, ANG-(1-7) and Mas receptor expression increases as the liver injury progresses [25,26]. These studies suggest that in liver disease the ACE2/ANG-(1-7)/Mas receptor axis is up-regulated in response to chronic damage. Support for a possible protective role of ANG-(1-7) in liver disease has been provided by an *in vivo* animal experiment in which the Mas receptor was pharmacologically inhibited [27]. In addition, Lubel et al. established that ANG-(1-7) ameliorates hepatic fibrosis in an *in vivo* animal model [28]. Furthermore, they demonstrated that plasma ANG-(1-7) levels are markedly elevated in human liver disease. Therefore, new actions of ANG-(1-7) are still being identified. These evidences remark that the physiological and pathophysiological implications of the ACE2/ANG-(1-7)/Mas receptor axis are broad.

In summary, we have demonstrated that acute administration of ANG-(1-7) stimulate the rapid phosphorylation of Akt in adipose tissue, liver and skeletal muscle, along with the phosphorylation of GSK-3β in liver and skeletal muscle of the rat *in vivo*, through a mechanism that appears to involve the Mas receptor. Our current results may shed light into the mechanism of action of ANG-(1-7) as a positive physiological contributor to insulin actions in extracardiac tissues.

Acknowledgements

FP Dominici is a Career Investigator from Consejo Nacional de Investigaciones Científicas y Tecnológicas of Argentina (CONICET), and received grant support from the University of Buenos Aires (UBA) and CONICET. JF Giani and MC Muñoz are research fellows from CONICET.

References

[1] Rao RH. Effects of angiotensin II on insulin sensitivity and fasting glucose metabolism in rats. Am J Hypertens 1994;7:655–60.
[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] Henriksen EJ. Improvement of insulin sensitivity by antagonism of the renin-angiotensin system. Am J Physiol Regul Integr Comp Physiol 2007;293:R974–80.
[4] Ferrario CM, Trask AJ, Jessup JA. Advances in biochemical and functional roles of angiotensin-converting enzyme 2 and angiotensin-(1-7) in regulation of cardiovascular function. Am J Physiol Heart Circ Physiol 2005;289:H2281–90.
[5] Santos RA, Ferreira AJ, Simoes ESAC. Recent advances in the angiotensin-converting enzyme 2-angiotensin(1-7)-Mas axis. Exp Physiol 2008;93:519–27.
[6] Iusuf D, Henning RH, van Gilst WH, Roks AJ. Angiotensin-(1-7): pharmacological properties and pharmacotherapeutic perspectives. Eur J Pharmacol 2008;585: 303–12.
[7] 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.
[8] 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.
[9] Dias-Peixoto MF, Santos RA, Gomes ER, Alves MN, Almeida PW, Greco L, et al. Molecular mechanisms involved in the angiotensin-(1-7)/Mas signaling pathway in cardiomyocytes. Hypertension 2008;52:542–8.
[10] 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.
[11] Giani JF, Mayer MA, Munoz MC, Silberman EA, Hocht C, Taira CA, et al. 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.

[12] Vaz-Silva J, Carneiro MM, Ferreira MC, Pinheiro SV, Silva DA, Silva-Filho AL, et al. The vasoactive peptide angiotensin-(1-7), its receptor Mas and the angiotensin-converting enzyme type 2 are expressed in the human endometrium. Reprod Sci 2009;16:247–56.
[13] Paul M, Poyan Mehr A, Kreutz R. Physiology of local renin-angiotensin systems. Physiol Rev 2006;86:747–803.
[14] Kudoh A, Matsuki A. Effects of angiotensin-converting enzyme inhibitors on glucose uptake. Hypertension 2000;36:239–44.
[15] 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.
[16] Munoz 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.
[17] Santos RA, Ferreira AJ, Pinheiro SV, Sampaio WO, Touyz R, Campagnole-Santos MJ. Angiotensin-(1-7) and its receptor as a potential targets for new cardiovascular drugs. Expert Opin Investig Drugs 2005;14:1019–31.
[18] 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.
[19] Ferrario CM. Angiotensin-converting enzyme 2 and angiotensin-(1-7): an evolving story in cardiovascular regulation. Hypertension 2006;47:515–21.
[20] Santos RA, Simoes 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 U S A 2003;100:8258–63.
[21] Taniguchi CM, Emanuelli B, Kahn CR. Critical nodes in signalling pathways: insights into insulin action. Nat Rev Mol Cell Biol 2006;7:85–96.
[22] Metzger R, Bader M, Ludwig T, Berberich C, Bunnemann B, Ganten D. Expression of the mouse and rat Mas proto-oncogene in the brain and peripheral tissues. FEBS Lett 1995;357:27–32.
[23] 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.
[24] Lee J, Kim MS. The role of GSK3 in glucose homeostasis and the development of insulin resistance. Diabetes Res Clin Pract 2007;77(Suppl 1):S49–57.
[25] Herath CB, Warner FJ, Lubel JS, Dean RG, Jia Z, Lew RA, et al. Upregulation of hepatic angiotensin-converting enzyme 2 (ACE2) and angiotensin-(1-7) levels in experimental biliary fibrosis. J Hepatol 2007;47:387–95.
[26] Paizis G, Tikellis C, Cooper ME, Schembri JM, Lew RA, Smith AI, et al. Chronic liver injury in rats and humans upregulates the novel enzyme angiotensin converting enzyme 2. Gut 2005;54:1790–6.
[27] Pereira RM, Dos Santos RA, Teixeira MM, Leite VH, Costa LP, da Costa Dias FL, et al. The renin-angiotensin system in a rat model of hepatic fibrosis: evidence for a protective role of angiotensin-(1-7). J Hepatol 2007;46:674–81.
[28] Lubel JS, Herath CB, Tchongue J, Grace J, Jia Z, Spencer K, et al. Angiotensin 1-7, an alternative metabolite of the renin-angiotensin system, is upregulated in human liver disease and has antifibrotic activity in the bile duct ligated rat. Clin Sci (Lond); 2009.