Angiotensin-(1–7) stimulates the phosphorylation of JAK2, IRS-1 and Akt in rat heart in vivo: role of the AT₁ and Mas receptors

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Giani JF, Gironacci MM, Muñoz MC, Peña 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 293: H1154–H1163, 2007. First published May 11, 2007; doi:10.1152/ajpheart.01395.2006.—Angiotensin (ANG) II exerts a negative modulation on insulin signal transduction that might be involved in the pathogenesis of hypertension and insulin resistance. ANG-(1–7), an endogenous heptapeptide hormone formed by cleavage of ANG I and ANG II, counteracts many actions of ANG II. In the current study, we have explored the role of ANG-(1–7) in the signaling crosstalk that exists between ANG II and insulin. We demonstrated that ANG-(1–7) stimulates the phosphorylation of Janus kinase 2 (JAK2) and insulin receptor substrate (IRS)-1 in rat heart in vivo. This stimulating effect was blocked by administration of the selective ANG type 1 (AT₁) receptor blocker losartan. In contrast to ANG II, ANG-(1–7) stimulated cardiac Akt phosphorylation, and this stimulation was blunted in presence of the receptor Mas antagonist A-779 or the phosphatidylinositol 3-kinase (PI3K) inhibi- torentort wortmannin. The specific JAK2 inhibitor AG-490 blocked ANG-(1–7)-induced JAK2 and IRS-1 phosphorylation but had no effect on ANG-(1–7)-induced phosphorylation of Akt, indicating that activation of cardiac Akt by ANG-(1–7) appears not to involve the recruitment of JAK2 but proceeds through the receptor Mas and involves PI3K. Acute in vivo insulin-induced cardiac Akt phosphorylation was inhibited by ANG II. Interestingly, coadministration of insulin with an equimolar mixture of ANG II and ANG-(1–7) reverted this inhibitory effect. On the basis of our present results, we postulate that ANG-(1–7) could be a positive physiological contributor to the actions of insulin in heart and that the balance between ANG II and ANG-(1–7) could be relevant for the association among insulin resistance, hypertension, and cardiovascular disease.

Angiotensin II; Janus kinase; insulin receptor substrate-1; signal transduction

ANGIOTENSIN II IS A MEMBER OF THE renin-angiotensin system (RAS), which initiates signals that result in the increase of blood pressure and also has an important role in cardiovascular and neuroendocrine physiology and electrolyte homeostasis (14, 15, 44). Angiotensin (ANG)-(1–7), a heptapeptide hormone formed by cleavage of ANG I by prolyl endopeptidases and neutral endopeptidases and also from ANG II by numerous enzymes, with angiotensin-converting enzyme homolog ACE2 being the most relevant (14, 15). ANG-(1–7) plays a major role in a counterregulatory arm within the RAS, because it opposes many of the actions of ANG II by inducing vasodilatation, diuresis, and natriuresis and also by inhibiting cell growth and norepinephrine release (14, 15, 24, 44). In addition, there is evidence indicating that ANG-(1–7) enhances the actions of bradykinin (36).

In recent years, a series of studies has revealed a clear connection between the signal transduction pathways that mediate insulin and ANG II actions in target tissues (18, 19, 51, 52). Insulin transmits its signal to the inner part of the cell through the insulin receptor (IR), a tetrameric protein with intrinsic tyrosine kinase activity (54). Upon insulin binding, the IR becomes activated, leading to the tyrosine phosphorylation of several intracellular proteins, including insulin receptor substrate (IRS)-1 and IRS-2 (50, 54). Many of the effects of insulin are mediated by activation of the enzyme phosphatidylinositol 3-kinase (PI3K) and downstream signaling pathways, including protein kinase B (Akt) (54). In addition, both the IR and the ANG type 1 receptor (AT1R) have been shown to activate the enzyme JAK2, a soluble nonreceptor tyrosine kinase that plays an important role in the transduction of the signals initiated by insulin and ANG II (42, 50, 51). Following PI3K stimulation, the serine (Ser)/threonine (Thr) kinase Akt becomes phosphorylated at Thr308 and Ser473, which results in its activation (2). This event is a necessary requirement for insulin to exert their metabolic and vascular effects (54).

ANG II negatively modulates insulin signaling at multiple levels, such as the IR, IRS-1 and IRS-2, PI3K, and Akt through an AT1R-mediated mechanism (5, 7, 18, 19, 48, 51, 52). This results in inhibition of the vasodilator and glucose transport properties of insulin (35, 38, 39, 51). Accordingly, selective blockade of the AT1R or inhibition of ACE improves insulin sensitivity (20, 21, 25, 27, 30) and enhances the response to insulin at various steps of the insulin signaling cascade (8, 9, 34, 48), suggesting that this therapy could prevent development of type 2 diabetes in patients with risk factors (27). This evidence clearly indicates that the signaling cross talk between insulin and ANG II has important physiological relevance.

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 (15, 26, 44). The counterregulatory effects of ANG-(1–7) on the pressor and trophic actions of ANG II appear to be mediated by the Mas receptor (MasR), a G protein-coupled receptor present in several tissues, including heart and kidney (16, 44). Nevertheless, a number of studies have shown that at pharmacological doses, ANG-(1–7) also interacts with the AT1R and the ANG type 2 receptor (AT2R)
(14, 15, 23). However, the intracellular signaling mechanisms of ANG-(1–7) are largely unknown.

The heart is one of the main targets for ANG-(1–7) actions (15), and since it is an insulin-responsive organ, disorders of insulin action, such as diabetes and obesity, can have profound effects on cardiac performance (1). Insulin signaling influences numerous functions within the heart, such as metabolic substrate preference, cell size, and the response of the heart to ischemia and hypertrophy (1). In the present study, we evaluated the ability of ANG-(1–7) to stimulate the phosphorylation of molecules involved in insulin signaling (namely, IR, JAK2, IRS-1, and Akt) in rat heart in vivo. Results were compared with those obtained with ANG II under the same conditions. Using selective receptor antagonists, we also analyzed the participation of the AT1R, AT2R, and MasR in the signaling pathways utilized by ANG-(1–7) in the heart. The involvement of the enzymes JAK2 and PI3K was analyzed by using the specific inhibitors tyrphostin AG-490 and wortmannin, respectively. In addition, we explored the effects of simultaneous injection of ANG-(1–7) and/or ANG II on the insulin-stimulated phosphorylation of Akt in this organ.

METHODS

Materials. The peptides ANG II, ANG-(1–7), and [7-β-Ala]ANG-(1–7) (A-779) were synthesized in our laboratory as described previously (24). The reagents and apparatus for SDS-PAGE and immunoblotting were obtained from Bio-Rad (Hercules, CA). The monoclonal anti-phosphotyrosine antibody (anti-PY; PY99), the polyclonal anti-IR β-subunit antibody (anti-IR; C-19), goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP), and goat anti-mouse IgG-HRP secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal anti IRS-1 antibody and the polyclonal anti-JAK2 antibody were purchased from Upstate Biotechnology (Lake Placid, NY). Phospho-Akt (Ser473) mouse monoclonal antibody that detects endogenous levels of Akt only when phosphorylated at Ser473 and the polyclonal Akt antibody that detects endogenous levels of total Akt1, Akt2, and Akt3 proteins (anti-Akt) were purchased from Cell Signaling (Beverly, MA). Enhanced chemiluminescence (ECL) was obtained from Amersham (Piscataway, NJ). The specific JAK2 inhibitor tyrphostin AG-490 was purchased from LC Laboratories (Woburn, MA). The remaining reagents, including the selective PI3K inhibitor wortmannin, were purchased from Sigma Chemical (St. Louis, MO).

Animals. Male Sprague-Dawley rats at 8 wk of age were used. Animals were housed in a controlled environment with a photoperiod of 12 h light and 12 h dark (lights on from 0600 to 1800) and a temperature of 20°C ± 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 Animal Studies Committee of the School of Pharmacy and Biochemistry, University of Buenos Aires, Argentina.

Surgical procedures, hormone administration, and tissue preparation. After an overnight fasting (14 h), rats were 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, and in vivo stimulation of the heart was obtained by the injection of 200-μl solutions containing either normal saline (0.9% NaCl), insulin (8 pmol/kg), ANG II (0.08–800 pmol/kg), ANG-(1–7) (0.08–800 pmol/kg), or equimolar (8 pmol/kg) mixtures of insulin and ANG II, insulin and ANG-(1–7), or insulin, ANG II, and ANG-(1–7) into the vena cava. At the indicated time points, the entire heart was removed and kept at −80°C until analysis.

For selective antagonism of the AT1R, losartan (10 mg/kg ip) was administered 30 min before intravenous administration of ANG II or ANG-(1–7). For selective antagonism of the AT2R or the MasR, PD-123319 (80 pmol/kg) or A-779 (80 pmol/kg) was used, respectively, and coadministered with either ANG II or ANG-(1–7) intravenously.

To inhibit JAK2 activity, AG-490 was dissolved in dimethyl sulfoxide (DMSO) and administered (80 nmol/kg ip) 30 min before administration of the hormones. After 5 min, the entire heart was removed and kept at −80°C until analysis.

To inhibit PI3K activity, wortmannin was dissolved in DMSO and administered intraperitoneally at a dose (1 mg/kg) considered to give rise to circulating concentrations of wortmannin that are selective for PI3K inhibition (4). Five minutes later, ANG-(1–7) was administered via vena cava, and after 5 min the entire heart was removed and kept at −80°C until analysis.

Tissue samples were homogenized in 10 volumes of a solubilization buffer containing 1% Triton together with phosphatase and protease inhibitors as described previously (3, 13). Heart 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 (3, 13).

Immunoprecipitation and immunoblotting. Equal amounts of solubilized heart protein (4 mg) were incubated at 4°C overnight with anti-IR, anti-IRS-1, or anti-JAK2 to a final concentration of 4 μg/ml for all antibodies. Immune complexes were collected by incubation with protein A-Sepharose 6 MB as described previously (3, 13, 34). SDS-PAGE and Western transfer of proteins to polyvinylidene difluoride membranes were performed as previously described (3, 34). Membranes were blocked by incubation for 2 h with a blocking buffer composed of Tris-buffered saline-Tween 20 (TBS-T) buffer [10 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.02% Tween 20 containing 3% BSA]. The membranes were then incubated overnight with anti-PY (1 μg/ml) to detect tyrosine phosphorylation or with anti-IR (1 μg/ml), anti-IRS-1 (1 μg/ml), or anti-JAK2 (1 μg/ml) to determine protein abundance. Finally, membranes were incubated for 1 h with goat anti-mouse IgG-HRP secondary antibody (for tyrosine phosphorylation detection) or goat anti-rabbit IgG-HRP (for protein detection). Specific bands were detected by ECL, and their intensities were quantitated by digital densitometry.

To determine the phosphorylation levels of Akt at Ser473, equal amounts of solubilized proteins (80 μg) were denatured by being boiled in reducing sample buffer, resolved by SDS-PAGE, and subjected to immunoblotting with anti-phospho-Akt (1:1,000 dilution). Cardiac Akt abundance was detected by incubation of the membranes with the anti-Akt antibody. Specific bands were detected by ECL, and their intensities were quantitated by digital densitometry.

Statistical analysis. Data were analyzed with analysis of variance (ANOVA) followed by the Tukey-Kramer test, using GraphPad InStat version 4.0.0 for Windows by GraphPad Software (San Diego, CA). A value of P < 0.05 was considered significant. All values are means ± SE.

RESULTS

ANG-(1–7) stimulates phosphorylation of JAK2 and IRS-1 in rat heart. Acute in vivo administration of ANG II (8 pmol/kg) induced the tyrosine phosphorylation of JAK2 and IRS-1 in rat heart, reaching a peak at 5 min (Fig. 1, A and B, top blots; n = 4). Time course experiments performed with ANG-(1–7) at the same dose showed that this peptide induced a noticeable stimulation of the tyrosine phosphorylation of JAK2 and IRS-1.
in rat heart that reached a maximal level at 5 min for both proteins (Fig. 1, A and B, top blots; n = 4). To provide a deeper comparison of the stimulating effects of ANG II and ANG-(1–7) on JAK2 and IRS-1 tyrosine phosphorylation, we performed a dose-dependent analysis. The whole heart was extracted 5 min after the intravenous administration of the peptides. A very similar pattern of stimulation was found for both hormones (Fig. 1, C and D, top blots). The maximal response was obtained with an 8 pmol/kg dose of either ANG II or ANG-(1–7). Noticeably, little or no effect was found with an 800 pmol/kg dose of either hormone (Fig. 1, C and D, top blots). Quantitation of multiple experiments revealed that ANG II at an 8 pmol/kg dose induced an approximately three- to fourfold increase in the tyrosine phosphorylation of JAK2 (Fig. 1, C and D). Under the same conditions, ANG-(1–7) increased the tyrosyl phosphorylation of JAK2 and IRS-1 by approximately three- to fourfold (Fig. 1, C and D; n = 4). The amount of immunoprecipitated protein was similar in all cases, as confirmed by reblotting the same membranes with anti-JAK2 or anti-IRS-1 (Fig. 1, bottom blots).

ANG-(1–7) induces phosphorylation of Akt in rat heart in vivo. To investigate whether ANG-(1–7) induced the activation of Akt, we evaluated effects of acute in vivo ANG-(1–7) administration on Akt phosphorylation in rat heart. As shown in Fig. 2, ANG-(1–7) induced the phosphorylation of Akt at Ser473, which peaked 5 min after injection of an 8 pmol/kg dose of the hormone (Fig. 2A, top blot). A similar level of phosphorylation was found within 10 min of stimulation (Fig. 2A, top blot). Unlike what was found for JAK2 and IRS-1, very similar levels of ANG-(1–7)-induced Akt phosphorylation were attained at both 8 and 800 pmol/kg (Fig. 2B, top blot). Under the same conditions, we found that ANG II was unable to activate Akt in rat heart (Fig. 2, A and B). Total protein was analyzed in each case by subjecting the corresponding heart extracts to immunoblotting with anti-Akt antibody (Fig. 2, bottom blots).

To determine whether the ANG-(1–7)-stimulating effects were mediated by the IR, we performed time course and dose dependence experiments. During the time frame analyzed (1 to 10 min), IR tyrosine phosphorylation was not modified after stimulation with 8 pmol/kg of either ANG II or ANG-(1–7). The dose dependence experiment involved administration of ANG II or ANG-(1–7) in a dose range of 0.08–800 pmol/kg. IR phosphorylation was not stimulated by either ANG-(1–7) or ANG II even at the highest dose utilized (data not shown).

Characterization of the receptors involved in acute effects exerted by ANG-(1–7) and ANG II in rat heart in vivo. Three different receptor blockers were used to characterize the participation of AT1R, AT2R, and MasR in the previously observed effects. Angiotensin II or ANG-(1–7) were injected at an 8 pmol/kg dose via the vena cava, and 5 min later, hearts were extracted and homogenized. Solubilized hearts proteins were subjected to immunoprecipitation with anti-JAK2 or anti-IRS-1 antibodies. As shown in Fig. 3, A and B, top blots, the AT1R antagonist losartan blocked the stimulating effects of ANG II and ANG-(1–7) on JAK2 and IRS-1 tyrosine phosphorylation. In contrast, the stimulating effects of ANG II and ANG-(1–7) on JAK2 and IRS-1 tyrosine phosphorylation were not blocked by either the AT2R antagonist PD-123319 (Fig. 3, C and D, top blots) or the MasR antagonist A-779 (Fig. 3, E and F, top blots).

As shown in Fig. 3G, the administration of the inhibitors by itself did not affect the phosphorylation of either JAK2 or IRS-1. The amount of protein immunoprecipitated was similar in all cases, as confirmed by reblotting the same membranes with anti-JAK2 or with anti-IRS-1 (Fig. 3, bottom blots).

Subsequently, we evaluated the role of the AT1R, AT2R, and MasR in the ANG-(1–7)-stimulated phosphorylation of Akt. As mentioned before, ANG II did not stimulate Akt phosphorylation, whereas ANG-(1–7) induced an approximately threefold increase in Akt serine phosphorylation (Fig. 4). As shown in Fig. 4, neither losartan nor PD-123319 modified the ANG-(1–7)-induced phosphorylation of Akt (Fig. 4, A and B, top blots). In contrast to what was observed for JAK2 and IRS-1, A-779 blocked ANG-(1–7) acute effects on Akt stimulation (Fig. 4C, top blot). The administration of the antago-
Interestingly, this treatment inhibited the stimulation of tyrosine phosphorylation of IRS-1 by both hormones (Fig. 5B, top blot), suggesting the direct involvement of JAK2 in this phenomenon. Protein abundance remained unchanged, as confirmed by reblotting same membranes with anti-JAK2 or anti-IRS-1 (Fig. 5, A and B, bottom blots). As shown in Fig. 5C, top blot, treatment with AG-490 did not affect Akt activation by ANG-(1–7), suggesting that JAK2 is not involved in the stimulation of Akt by ANG-(1–7). Administration of DMSO alone did not modify the parameters analyzed (data not shown). The phosphorylation levels of JAK2, IRS-1, and Akt were not affected by the administration of the JAK2 inhibitor alone (Fig. 5D).

ANG-(1–7)-stimulated phosphorylation of Akt is mediated by PI3K. To determine whether the ANG-(1–7)-induced Akt phosphorylation in rat heart is mediated by PI3K, rats were pretreated with wortmannin (1 mg/kg, 5 min), and effects on Akt phosphorylation (Ser473) were determined. As shown in Fig. 6, wortmannin inhibited ANG-(1–7)-stimulated Ser473 phosphorylation of Akt (n = 4). Phosphorylation levels of Akt in hearts from rats that were pretreated with wortmannin alone were not statistically different from those obtained after administration of vehicle (DMSO) (data not shown).

In vivo interactions among ANG II, ANG-(1–7), and insulin. To explore the interaction between ANG II and ANG-(1–7) signaling pathways and their influence in insulin signaling in rat heart, normal rats were acutely stimulated with insulin, ANG II, or ANG-(1–7) or simultaneously with insulin and ANG II, with insulin and ANG-(1–7), or with a combination of these three hormones via the vena cava. After 5 min, hearts were extracted and homogenized. Total heart extracts were submitted to immunoblotting with anti-phospho Akt antibodies. As shown in Fig. 7, insulin (8 pmol/kg) stimulated the phosphorylation of Akt approximately threefold. The acute administration of ANG II alone (8 pmol/kg) did not stimulate Akt phosphorylation (Ser473) were determined. As shown in Fig. 6, wortmannin inhibited ANG-(1–7)-stimulated Ser473 phosphorylation of Akt (n = 4). Phosphorylation levels of Akt in hearts from rats that were pretreated with wortmannin alone were not statistically different from those obtained after administration of vehicle (DMSO) (data not shown).

DISCUSSION

Cross talk between the RAS and the insulin signaling system has drawn great attention, because hypertension and insulin resistance often coexist and are primary risk factors for cardiovascular disease (11, 49). Insulin resistance and hyperinsulinemia are closely associated with several disease processes such as hypertension, non-insulin-dependent diabetes, neointimal hyperplasia, and dyslipidemia (11, 49). However, the pathogenic role of insulin resistance and/or hyperinsulinemia in the development of hypertension and other cardiovascular diseases is still not clear. Studies have shown that ANG II infusion induces insulin resistance as well as a substantial reduction in glucose disposal in normal skeletal muscle (35, 38, 39) and that treatment with both AT1R blockers and ACE inhibitors enhances insulin sensitivity (20, 21, 27, 30, 34, 48, 51). Therefore, overactivity of the RAS as observed in cardiovascular diseases is likely to impair insulin signaling and contribute to insulin resistance (49).
Fig. 3. Effects of losartan, PD-123319, and A-779 on JAK2 and IRS-1 tyrosine phosphorylation in rat heart in vivo. Rats received saline (−) or losartan (10 mg/kg ip; A and B) 30 min before acute administration of 0.2 ml of a solution of normal saline or solutions of normal saline containing ANG II (8 pmol/kg) or ANG-(1–7) (8 pmol/kg) via the inferior vena cava. In experiments using PD-123319 (C and D) or A-779 (E and F), the antagonists were simultaneously administered with the hormones at 80 pmol/kg. In all cases, hearts were removed 5 min after stimulation and homogenized as described in METHODS. Solubilized heart proteins were subjected to IP with anti-JAK2 or anti-IRS-1, followed by IB with PTyr. To determine protein abundance, membranes were reprobed with anti-JAK2 or anti-IRS-1. The effect of administration of each antagonist by itself is shown in G and was compared with that in animals which received vehicle only (−). Data are means ± SE, expressed as relative increases in phosphorylation over the basal value (n = 4 for every experiment). *P < 0.05 vs. basal value.
Emerging evidence suggests that the RAS is composed of two distinct arms. One arm of this biochemical axis represents the classically accepted hypertensive pathway in which ANG II is formed from ANG I by ACE, whereas the other arm of the system acts as an antihypertensive pathway forming ANG-(1–7) (15, 16, 44). The heptapeptide ANG-(1–7) is formed by two major pathways. In one of them, the common precursor ANG I is cleaved to generate ANG-(1–7) by the enzymes prolyl endopeptidase, endopeptidase 24.11, and thimet oligopeptidase (15, 16). The other, newly discovered pathway involves the participation of ACE2 that efficiently hydrolyzes ANG II into ANG-(1–7) (15, 16). Although the receptor site of action of ANG-(1–7) is still a matter of controversy, the peptide is an endogenous ligand for the G protein-coupled receptor Mas (45) that, besides binding ANG-(1–7) with high affinity, has been shown to be a physiological antagonist of the AT1R (29).

It has been consistently demonstrated that following injection of ANG II into rats, there is a rapid tyrosine phosphorylation of the major insulin receptor substrates (IRS-1 and IRS-2) in the heart. (7, 41, 51, 52). This phenomenon apparently involves JAK2, which associates with the AT1R and IRS-1/IRS-2 after ANG II stimulation (7, 51). ANG II-induced phosphorylation leads to binding of PI3K to IRS-1 and IRS-2 (51). However, in contrast to other ligands, ANG II injection results in an acute inhibition of insulin-stimulated PI3K activity, which acts as an essential transducer of the metabolic actions of insulin (51). Reduced tyrosyl phosphorylation as well as increased phosphorylation of the β-subunit of the IR and IRS-1 in inhibitory serine sites were proposed as two possible mechanisms involved in this attenuation (19, 51). Thus, considering that ANG-(1–7) has been shown to counteract many of the actions of ANG II, including vasoconstriction and proliferation, the main objective of this study was to investigate the participation of ANG-(1–7) in the cross talk between the insulin and ANG II signaling system. We used the heart, since this organ has been identified as one of the main targets for ANG-(1–7) actions (15). Both ACE2 and receptor Mas are present in the heart; thus the ANG-(1–7)-Mas axis assumes a key role for understanding the actions of cardiac RAS (31, 44).

Our first goal was to determine whether ANG-(1–7) was able to induce the phosphorylation of molecules that participate in the signaling of insulin in rat heart in vivo. To that end, we analyzed the in vivo effect of an acute administration of this peptide on the phosphorylation of molecules known to participate in the insulin signaling pathway. We proved that ANG-(1–7) stimulates the tyrosine phosphorylation of IRS-1 in vivo. We have shown that both the time and concentration necessary to attain the maximal stimulating response were similar to those previously described for ANG II (7, 41). This reinforces the previously postulated concept that the IRS proteins serve as a convergence site for the signal transduction of several hormones (12). In addition, we have demonstrated that this stimulating effect of...
ANG-(1–7) is not mediated by the IR kinase and that it appears to be mediated by JAK2. This nonreceptor tyrosine kinase has been shown to be activated and associated with the AT1R in response to ANG II both in vitro (32) and in vivo (42). The fact that the ANG-(1–7)-induced tyrosine phosphorylation levels of JAK2 correlated with those of IRS-1 suggested that the stimulatory effect of ANG-(1–7) on cardiac IRS-1 phosphorylation was most likely attributed to the ANG-(1–7)-induced activation of JAK2 through an AT1R-related mechanism. This hypothesis was confirmed through the use of the selective AT1R antagonist losartan, which abolished ANG-(1–7)-induced phosphorylation of both JAK2 and IRS-1. The use of selective antagonists PD-123319 and A-779 indicated that AT2R and/or MasR receptor appears not to participate in this stimulating effect of ANG-(1–7).

Another important observation reported in this study is that ANG-(1–7) neutralizes the inhibitory effects of ANG II on the insulin-induced activation of Akt. However, the mechanism of this neutralizing effect is not clear and deserves further exploration.

Besides being a specific ligand for the MasR, ANG-(1–7) also interacts with the AT1R (23, 44), and some of its actions can be blocked by AT1R blockers such as losartan. This agrees with our observation that losartan blocked the ANG-(1–7)-induced phosphorylation of JAK2 and IRS-1. When ANG-(1–7) was injected in rats that were pretreated with the specific JAK2 inhibitor AG-490, ANG-(1–7)-induced IRS-1 phosphorylation was abolished (43), which demonstrated that in human endothelial cells, ANG-(1–7), through receptor Mas, stimulates endothelial nitric oxide synthase activation and nitric oxide production via Akt-dependent mechanisms. Together with that report, our current findings reinforce the notion that the ANG-(1–7)-MasR axis is important for cardiovascular function. To our knowledge, this study is the first to characterize ANG-(1–7) signal transduction in rat heart in vivo.

**Fig. 5. Effects of AG-490 on ANG II- and ANG-(1–7)-induced tyrosine phosphorylation of JAK2 and IRS-1 and Akt Ser473 phosphorylation in rat heart in vivo.** Rats were treated with saline (–) or AG-490 (0.1 ml, 80 nmol/kg). After 30 min, the animals received acutely a single intravenous dose (0.2 ml) of a solution of normal saline (–) or solutions of normal saline containing either ANG II (8 pmol/kg) or ANG-(1–7) (8 pmol/kg). After 5 min, hearts were removed and homogenized as described in METHODS. Solubilized heart proteins were subjected to IP with anti-JAK2 (A) or anti-IRS-1 (B) followed by IB with PTyr. To determine protein abundance, membranes were reprobed with anti-JAK2 or anti-IRS-1. C: solubilized heart proteins were subjected to IB with Akt-PSer. Data are means ± SE, expressed as relative increases in phosphorylation over the basal value (n = 4). *P < 0.05 vs. basal value. Akt protein abundance was determined by IB with the anti-Akt antibody. D: effect on Akt phosphorylation of AG-490 administered alone.

**A:** IP: JAK2

**B:** IP: IRS-1

**C:** IP: Akt-PSer

**D:** IP: JAK2

**E:** IP: IRS-1

**F:** IP: Akt-PSer

**G:** Solubilized heart proteins were subjected to IP and IB with Akt-PSer. Data are means ± SE, expressed as relative increases in phosphorylation over the basal value (n = 4). *P < 0.05 vs. basal value. Akt protein abundance was determined by IB with the anti-Akt antibody. D: effect on Akt phosphorylation of AG-490 administered alone.
ylation in the heart was abolished, univocally demonstrating the involvement of JAK2 in this signaling event. At the same time, we detected that ANG-(1–7)-induced activation of Akt was unaltered by AG-490, implying that JAK2 does not participate in this signaling event.

In PC12W cells that exclusively express AT2R and not AT1R, activation of the AT2R by ANG II has been shown to inhibit insulin-induced PI3K activity and Akt phosphorylation (10), suggesting a potential negative role for AT2R in the crosstalk between ANG II and insulin. Although some of the effects reported for ANG-(1–7) are mediated by AT2R (15, 22, 44, 53), in the present work we have shown that blockade of the AT2R with the selective antagonist PD-123319 did not alter ANG-(1–7)- or ANG II-induced phosphorylation of JAK2, IRS-1, or Akt in rat heart, suggesting that AT2Rs are not involved in such responses. Hence, it appears that tissue and species differences could be critical for ANG-(1–7) actions.

The observation that ANG-(1–7) is as potent as ANG II in stimulating the phosphorylation of JAK2 and IRS-1 in the rat heart (current results) is in keeping with findings that the heptapeptide is as potent as ANG II in increasing the release of norepinephrine in rat atria (22) and releasing vasopressin from the rat hypothalmo-neurohypophyseal system (46), as well as eliciting cardiovascular effects when injected into the dorsal medulla of rats (6). It has been reported that ANG-(1–7) binds 150 times less effectively than native ANG II in rat brain zones, where AT1R is predominant (40), and 10 times less effectively than ANG II in rat renal cortex (23). However, to date there is no report about ANG-(1–7) affinity for AT1Rs in the heart, so we cannot disregard the possibility that ANG-(1–7) may bind to heart AT1Rs with affinity similar to that of ANG II and in this way stimulate IRS-1 and JAK2 phosphorylation. Another possibility is that AT1R and MasRs may interact directly through heterodimer formation, as previously demonstrated (29).

By acute administration of mixtures of hormones, we determined that ANG II attenuated the insulin-stimulated phosphorylation of Akt at Ser473 in rat heart. This result agrees with previously reported observations (5, 7). Interestingly, when ANG-(1–7) was administered simultaneously with ANG II and insulin, a clear restitution of the insulin-stimulated phosphorylation of Akt at Ser473 was observed, showing that ANG-(1–7) ameliorates the detrimental effects exerted by ANG II at this level. The phosphorylation levels of Akt attained after the coadministration of insulin and ANG-(1–7) were similar to those detected after acute stimulation with insulin alone, suggesting that the ability of ANG-(1–7) to restitute insulin-stimulated Akt activation in the presence of ANG II is not the consequence of a potentiation of the stimulating capacity of insulin.

In conclusion, we have demonstrated that acute ANG-(1–7) administration leads to stimulation of JAK2, IRS-1, and Akt in rat heart in vivo. ANG-(1–7)-induced IRS-1 phosphorylation involves activation of JAK2 that is mediated by an AT1R-related mechanism. In addition, we have shown that acute administration of ANG-(1–7) stimulates the rapid phosphorylation of Akt in rat heart in vivo through the MasR by a PI3K-dependent mechanism. Finally, we have demonstrated that ANG-(1–7) can reverse the detrimental effects of ANG II on the insulin-stimulation of Akt in rat heart in vivo. On the basis of our current results, we postulate that ANG-(1–7) could be a positive physiological contributor to the actions of insulin in cardiac tissue.

The balance between ANG II and ANG-(1–7) could be relevant for the association among insulin resistance, hypertension, and cardiovascular disease. Thus our findings broaden...
the possibilities for treating cardiovascular diseases, indicating that agonists for the ANG-(1–7)-Mas axis could be of potential therapeutic use.

GRANTS

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