Published in final edited form as:

Neuropharmacology. 2014 June ; 81: 267–273. doi:10.1016/j.neuropharm.2013.06.022.

Angiotensin-[1-12] interacts with angiotensin type I receptors

King H. Chan^{a,§}, Yi H. Chen^{c,§}, Ying Zhang^{d,e}, Yung H. Wong^{a,b}, and Nae J. Dun^{d,*}

^aDivision of Life Science and Biotechnology Research Institute, Hong Kong University of Science and Technology Clear Water Bay, Kowloon, Hong Kong

^bState Key Laboratory of Molecular Neuroscience, Hong Kong University of Science and Technology Clear Water Bay, Kowloon, Hong Kong

^cGraduate Institute of Acupuncture Science, China Medical University, Taichung, Taiwan

^dDepartment of Pharmacology, Temple University School of Medicine, Philadelphia, PA 19140 USA

Abstract

Angiotensin-(1-12) [Ang-(1-12)], a newer member of angiotensin peptides, is proposed to be converted enzymatically to angiotensin I (Ang I) and to angiotensin II (Ang II); the latter being the bioactive peptide. We studied the Ang-(1-12) and Ang II responses in COS-7 cells or CHO cells transfected with 5 μ g AT₁R by monitoring [Ca²⁺]_i using the Fluo-4. Ang II (1 pM–1 μ M) and Ang-(1-12) (5 pM–5 μ M) increased [Ca²⁺]_i with an EC₅₀ of 0.19 nM and 24 nM in COS-7 cells; and 0.65 nM and 28.7 nM in CHO cells. The AT₁R antagonist losartan (1 nM–10 μ M) suppressed [Ca²⁺]_i induced by Ang-(1-12) and Ang II. In CHO cells transfected with 5 μ g AT₂R, Ang II (1 pM–1 μ M) increased [Ca²⁺]_i, with an EC₅₀ of 9.68 nM; whereas, Ang-(1-12) (5 pM–5 μ M) failed to elicit a significant change in [Ca²⁺]_i. In CHO cells transfected with AT₁R, Ang-(1-12) stimulated ERK phosphorylation with a potency 300-fold less than that of Ang II. To evaluate the activity of Ang-(1-12) on native AT₁R, whole cell patch recordings were made from neurons in the rat hypothalamic slices. Ang II or Ang-(1-12) ejected by pressure from a micropipette elicited a membrane depolarization; the latter was blocked by losartan (10 μ M), and not affected by the AT₂R antagonist PD 123319 (10 μ M), nor by the angiotensin converting enzyme inhibitor captopril (10 μ M). Our result shows that Ang-(1-12) may produce its biological activity by acting directly on AT₁R, albeit at a concentration higher than that of Ang II.

Keywords

ACE inhibitor; angiotensin II; G-protein; hypothalamus

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*Corresponding author: Nae J. Dun Department of Pharmacology, Temple University School of Medicine, 3420 N. Broad Street, Philadelphia, PA 19140 USA Tel: (1)215-707-3498 Fax: (1)215-707-7068 ndun@temple.edu.

§Authors contribute equally

^eCurrent address: Department of Pathophysiology, Kunming Medical University, Kunming, P R China

Conflict of interest: Authors declare no conflict of interest.

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1. Introduction

The renin-angiotensin system (RAS) consisting of three sequentially processed peptides: namely, angiotensinogen, angiotensin I (Ang I) and angiotensin II (Ang II), serves as a coordinated neurohumoral network whose proper function ensures homeostasis of an array of physiological activities such as body fluid regulation, electrolyte balance, and cardiovascular activity. Ang II is generally regarded to be the most active form of the three angiotensins. This fundamental concept relative to the operation of RAS has not changed, till the discovery of Ang (1-7), a heptapeptide derived from either Ang I or Ang II (Santos et al., 2003; Trask and Ferrario, 2007). The pharmacology of Ang (1-7) is uniquely different from that of Ang II in that the action of Ang (1-7), particularly with respect to the cardiovascular system, is to counterbalance the activity of Ang II (Ferrario et al., 1997). Further, Ang (1-7) interacts with the Mas receptor that is pharmacologically distinct from the angiotensin receptor type I (AT₁R) and angiotensin receptor type II (AT₂R), upon which Ang I and Ang II exert their effects (Santos et al., 2003; Trask and Ferrario, 2007). There is some evidence that Ang II is further metabolized to two smaller fragments, referred to as Ang III and Ang IV, which are shown to be bioactive (von Bohlen und Halboch, 2003). Ang IV interacts with a site that is distinctively different from AT₁R and AT₂R as demonstrated by a poor affinity to AT₁R and AT₂R, but with a high affinity to a site later named AT₄R (von Bohlen und Halboch, 2003).

More recently, the isolation and identification of angiotensin-(1-12), abbreviated Ang-(1-12), from the rat intestine by Nagata et al. (2006) has added another layer of complexity to the biology of RAS. A bolus intravenous administration of Ang-(1-12) to anesthetized rats increased blood pressure in a dose-dependent manner. Further, the *ex vivo* vasoconstrictor responses to lower doses (< 30 nmol/L) of Ang-(1-12) were prevented by prior administration of the angiotensin converting enzyme (ACE) inhibitor captopril or the AT₁R blocker CV-11974; a higher dose (100 nmol/L) of Ang-(1-12) could elicit a small vasoconstriction in the presence of captopril, but not CV-11974 (Nagata et al., 2006). On the basis of these findings, the vasoconstriction response to Ang-(1-12) is attributed to a rapid conversion of Ang-(1-12) in the circulation to Ang I and to Ang II; the latter being the bioactive peptide (Nagata et al., 2006). A more recent study shows that in a Langendorff apparatus, Ang-(1-12) when added to Krebs solution perfusing the isolated rat hearts, caused an appearance of Ang I and Ang II in the perfusate that peaked between 30 and 60 min of recirculation, supporting the concept that Ang(1-12) serves as a precursor for the formation of Ang I and Ang II in the heart (Trask et al., 2008).

Results from several recent studies suggest that the biological activity of Ang-(1-12) may not be as simple as initially proposed (Nagata et al., 2006), and that the metabolic pathway of Ang-(1-12) appears to be tissue- and enzyme-dependent (Jessup et al., 2008; Trask et al., 2008; Isa et al., 2009; Nagata et al., 2010; Pereira et al., 2012; Westwood and Chappell, 2012). Moreover, chronic intracerebroventricular administration of Ang-(1-12) antiserum significantly lowered systolic blood pressure without a concomitant change in plasma concentrations of Ang I, Ang II or Ang (1-7) in transgenic (mRen2)27 hypertensive rats as compared to that injected with pre-immune IgG, leading to the proposal that Ang-(1-12)

may be functionally active in the brain (Isa et al., 2009). Here, experiments were undertaken to explore the hypothesis that Ang-(1-12) may produce its biological activity by interacting directly with angiotensin receptors.

2. Materials and Methods

2.1 Cell culture and transfection

Chinese hamster ovary (CHO) cells stably expressing the 16z25 chimeric $G\alpha_q$ subunit (CHO/16z25) were generated and characterized as previously described (New and Wong, 2004). CHO/16z25 cells were maintained in F12 medium containing 10% (v/v) fetal bovine serum (FBS). COS-7 kidney fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% (v/v) FBS. In both cases, 50 units ml^{-1} penicillin and 50 $\mu\text{g ml}^{-1}$ streptomycin were incorporated in the growth medium, and the cells were cultured at 37°C in a humidified 5% CO_2 incubator. One day prior to transfection, CHO/16z25 or COS-7 cells were seeded in 100 mm plates at a density of 1×10^6 cells per plate. Cells were transiently transfected at 50-80% confluency with 5 μg type 1 or type 2 angiotensin II receptor cDNAs using LipofectAMINE PLUS reagents according to the manufacturer's instructions. After 24 h, transfected cells were seeded onto 96-well or 12-well plates for fluorometric or ERK phosphorylation assays, respectively.

2.2. Fluorometric assay for intracellular Ca^{2+} mobilization

Cells in normal growth medium were seeded into clear-bottomed black-walled 96-well plates at 15,000 cells per well one day before assay. Cells in each well were labeled with 2 μM Fluo-4 (Invitrogen, Carlsbad, CA) in 200 μl of Hank's balanced salt solution (pH 7.5) containing 2.5 mM probenecid for 1 h at 37°C prior to the addition of test compounds. To test for competitive interactions, cells were pre-incubated with buffer containing various concentrations of losartan for 30 min at 37°C before the assay. Ligand-induced changes in fluorescence were detected in the fluorometric imaging plate reader FLIPR^{TETRA}TM (Molecular Devices/MDS Analytical Technologies, Sunnyvale, CA) with an excitation wavelength of 488 nm as described previously (Liu et al., 2003). The real-time fluorescent signal was monitored for 3 min. Results were expressed as changes in relative fluorescence units (RFU). Concentration-response curves were generated by determining the maximal change in RFU of each data set. Numerical analysis of the statistics and EC_{50} (median effective concentration) values were performed on GraphPad Prism version 3.03.

2.3. Immunodetection of ERK phosphorylation

Cells were seeded into 12-well plates at 2×10^5 cells per well overnight and then subjected to serum withdrawal for 4 h to reduce the basal ERK phosphorylation. Test compounds were diluted in serum-free culture medium at desired concentrations and the cells were treated with the test compounds for 5 min in the presence or absence of losartan. Reactions were stopped by aspiration and then addition of 150 μl of lysis buffer with protease inhibitors (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 40 mM NaP_2O_7 , 1% Triton X-100, 1 mM dithiothreitol, 200 μM Na_3VO_4 , 100 μM phenylmethylsulfonyl fluoride, 2 $\mu\text{g/ml}$ leupeptin, 4 $\mu\text{g/ml}$ aprotinin, and 0.7 $\mu\text{g/ml}$ pepstatin). Cells were lysed for 30 min at 4°C with agitation, and the collected total cell lysates were cleared by centrifugation. Protein

samples were resolved in SDS-PAGE and transferred to nitrocellulose membranes using an iBlot system (Invitrogen). Phosphorylated ERK was detected using anti-phospho-ERK (Thr²⁰²/Tyr²⁰⁴) and anti-ERK antibodies from Cell Signaling Technology (Beverly, MA) as described previously (Liu et al., 2003). The immunoblots were visualized by chemiluminescence with an ECL kit (Amersham Biosciences), and the images detected in X-ray films were quantified by densitometric scanning using the Eagle Eye II still video system (Stratagene, La Jolla, CA).

2.4. Experimental animals

Young Sprague-Dawley rats 17-20 days old (Ace Animals Inc., Boyertown, PA) were used in electrophysiological experiments. Procedures used for obtaining 250 μ m coronal hypothalamic slices were similar to that described (Stern et al., 1999). Experimental protocols were reviewed and approved by the Temple University Institutional Animal Care and Use Committee, in accordance with the NIH Guide for the Care and Use of Laboratory Animals 1996. Animals were housed under a 12/12-h light/dark cycle with free access to food and water. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.5. Whole cell patch recording techniques

Rats of either sex were anesthetized with 4% isoflurane and decapitated. The hypothalamus was rapidly removed and sectioned to 250 μ m using a vibratome. Coronal hypothalamic slices were incubated in Krebs solution gassed with 95% O₂/5% CO₂ in a holding chamber at room temperature for one hour prior to commencing electrical recordings. One slice was placed between two pieces of nylon mesh in the recording chamber (< 0.5 ml) and superfused with a Krebs solution of following composition (in mM): 127 NaCl, 1.9 KCl, 1.2 KH₂PO₄, 2.4 CaCl₂, 1.3 MgCl₂, 26 NaHCO₃ and 10 glucose; which was saturated with 95% O₂ and 5% CO₂. All experiments were performed in room temperature (20 $\pm$ 1°C). Whole-cell patch recordings in current clamp mode were made from hypothalamic neurons located dorsomedial to the 3rd ventricle. Patch pipettes pulled from thin-walled fiber-filled borosilicate glasses (OD 2.0 mm, World Precision Instrument, Inc., Sarasota, FL) and filled with a solution of following composition (in mM): 130 K⁺ gluconate, 1 MgCl₂, 2 CaCl₂, 4 Mg²⁺ATP, 10 EGTA and 10 HEPES, had an input resistance of 2-5 M Ω . Signals were recorded using an Axopatch 1C (Axon Instruments, Foster City, CA), low-pass filtered at 2 kHz and acquired using a PC and pCLAMP software (version 10.2, Axon Instruments) for later analysis. Ang II (1 mM) or Ang-(1-12) (3 mM) was applied from a micropipette positioned above the recording neuron by a puff of nitrogen using the Picospritzer (General Valve Inc., Fairfield, NJ). Other pharmacological agents were dissolved in Krebs solution and applied to the hypothalamic slices by superfusion.

2.6. Chemicals and reagents

Angiotensin-(1-12), angiotensin I and angiotensin II were from Phoenix Pharmaceuticals, Inc. (Burlingame, CA 94010); losartan, captopril and PD123319 were from Sigma-Aldrich (St. Louis, MO). Other chemicals and reagents were obtained from Sigma-Aldrich, St. Louis, MO.

2.7. Statistical analysis

The level of statistical significance for differences between various drug treatments and pre-drug controls were determined by the two-tailed Student's *t*-test (control and experimental samples were compared from different groups of preparations) and by the Student's paired *t*-test (control and experimental samples were compared from the same groups of preparations). Differences were considered significant at $p < 0.05$.

3. Results

3.1. FLIPR assay

The molecular targets of Ang II are the AT₁R and AT₂R that are primarily coupled to G_q (Sasaki et al., 1991) and G_i (Zhang and Pratt, 1996) proteins, respectively. To facilitate the assessment of Ang-(1-12) on AT₁R and AT₂R, we utilized a recombinant cell line which can efficiently detect activation of both G_i- and G_q-coupled receptors (New and Wong, 2004). CHO/16z25 cells stably expressing a chimeric Gα₁₆ subunit (Mody et al., 2000) have been demonstrated to link the activation of G_i-coupled receptors to intracellular Ca²⁺ mobilization (Liu et al., 2003). Hence, Ca²⁺-based FLIPR assay was performed using the CHO/16z25 cells transiently expressing the human AT₁R or AT₂R. Transfected CHO/16z25 cells were challenged with increasing concentrations of Ang II or Ang-(1-12). Ang II (1 pM-1 μM) stimulated Ca²⁺ mobilization in AT₁R transfectants with an EC₅₀ value of 0.65 ± 0.07 nM (mean $\pm$ SEM; Fig. 1A). Although Ang-(1-12) also stimulated Ca²⁺ mobilization in AT₁R transfectants, its potency (EC₅₀ of 28.7 ± 2.3 nM) and maximal response (26% of the maximal Ang II response) were lower than those of Ang II (Fig. 1A). The response induced by Ang-(1-12) was dependent on the expression of AT₁R because untransfected CHO/16z25 cells did not respond to the ligand. In CHO/16z25 cells expressing the AT₂R, Ang II stimulated Ca²⁺ mobilization with an EC₅₀ value of 9.68 ± 3.40 nM (Fig. 1A). However, Ang-(1-12) failed to elicit a significant Ca²⁺ response even at a concentration as high as 5 μM (Fig. 1A). These results suggest that Ang-(1-12) may act as an AT₁R partial agonist.

Since AT₁R is coupled to G_q (Sasaki et al., 1991), we examined whether Ang-(1-12) can mobilize intracellular Ca²⁺ via endogenous G_q proteins. COS-7 cells transiently expressing the AT₁R were subjected to FLIPR assays in the presence of increasing concentrations of Ang II or Ang-(1-12). Similar to the results obtained with CHO/16z25 cells, both Ang II and Ang-(1-12) dose-dependently stimulated Ca²⁺ mobilization in the transfected COS-7 cells with EC₅₀ values of 0.19 ± 0.05 nM and 24 ± 3.7 nM, respectively (Fig. 1B). Again, Ang-(1-12) behaved as a partial agonist at the AT₁R with a maximal response less than 50% of that generated by Ang II (Fig. 1B).

3.2. Pharmacological property

We used losartan, an AT₁R-selective antagonist (Lankley et al., 1991; Timmermans et al., 1993) to confirm that the observed Ang-(1-12) response was indeed mediated via the AT₁R. CHO/16z25 cells expressing the AT₁R were stimulated with 5 μM Ang-(1-12) in the absence or presence of increasing concentrations (0.1-10 μM) of losartan. The Ca²⁺ response stimulated by Ang-(1-12) was effectively suppressed by losartan in a dose-dependent

manner (Fig. 1C). The magnitude of the inhibition was similar to that observed for Ang II (Fig. 1C).

As Ang II can induce the phosphorylation of extracellular signal-regulated protein kinases (ERK) through G_q , we examined whether Ang-(1-12) can elicit the same response. Indeed, Ang-(1-12) dose-dependently stimulated ERK phosphorylation in AT₁R transfectants (Fig. 2A). In agreement with the FLIPR assays, Ang-(1-12) was less potent than Ang II in eliciting ERK phosphorylation; the latter only became detectable at 300 nM of Ang-(1-12); whereas Ang II required a mere 1 nM (Fig. 2A). Both Ang-(1-12)- and Ang II-induced ERK responses were inhibited in the presence of losartan (Fig. 2B). Observed changes in ERK phosphorylation was not due to alterations in the expression of ERK because none of the treatments had any effect on the total amount of ERK (Fig. 2).

3.3. Whole cell patch recordings from single hypothalamic neurons

Ang II depolarizes neurons in the rat paraventricular nucleus via activation of AT₁R (Cato and Toney, 2005; Latchford and Ferguson, 2005). To validate the effect of Ang-(1-12) on native AT₁R, whole cell patch clamp recordings were performed on neurons of rat hypothalamic slices in a manner similar to that described (Cato and Toney, 2005; Latchford and Ferguson, 2005).

We first validated previous observations that neurons in the rat hypothalamus express AT₁R and the activation of which causes a membrane depolarization (Cato and Toney, 2005; Latchford and Ferguson, 2005). Ang II (1 mM), when applied to the recording neuron from a micropipette by a puff of nitrogen, evoked a membrane depolarization in 10 of the 27 hypothalamic neurons examined (Fig. 3A). The response varied between 4 and 12 mV, with a mean amplitude of 6.9 ± 0.9 mV (mean $\pm$ SEM; $n=10$). Depolarizations of similar amplitude could be repeatedly elicited, when the interval between Ang II applications was kept at 20-30 min (Fig. 3A). Ang II responses were suppressed by pretreatment of the slices with the AT₁R antagonist losartan (10 μ M; $n=4$), but not with the ACE inhibitor captopril (10 μ M; $n=3$); a representative experiment is shown in Fig. 3B and 3C. The result confirms the earlier observation that Ang II depolarizes hypothalamic neurons via activation of AT₁R (Cato and Toney, 2005; Latchford and Ferguson, 2005).

In an effort to achieve about the same magnitude of Ang II-induced depolarizations, the concentration of Ang-(1-12) in the micropipette was increased to 3 mM. Ang-(1-12) by pressure ejection elicited a membrane depolarization in 19 of the 49 hypothalamic neurons examined; the response varied between 4 and 13 mV, with a mean amplitude of 6.2 ± 0.6 mV ($n=19$). Similar to Ang II, repeated applications of Ang-(1-12) to hypothalamic neurons at intervals of 20-30 min elicited reproducible membrane depolarization. The depolarization was antagonized by prior superfusion of the slice with losartan (10 μ M), but not by PD123319 (10 μ M) (Fig. 4B, C, D). Pretreatment of the slices with captopril (10 μ M) for 30 min failed to suppress the Ang-(1-12) induced depolarization (Fig. 4A, D).

4. Discussion

Using intracellular Ca^{2+} mobilization as an index, our results showed that COS-7 and CHO cells transiently transfected with AT_1R cDNA responded to Ang II in a dose dependent manner with an EC_{50} of 0.19 nM and 0.65 nM, and that the response was suppressed by the AT_1R antagonist losartan, attesting to the pharmacological characteristic of transfected receptors being that of AT_1R . Ang-(1-12) also mobilized Ca^{2+} in COS-7 and CHO cells transfected with AT_1R cDNA in a concentration dependent manner, with an EC_{50} of 24 nM and 28.7 nM, which is 40- to 100-folds higher than that of Ang II. In addition, CHO cells transfected with AT_2R cDNA failed to respond to Ang-(1-12). Contrary to the finding in *ex vivo* rat aorta preparations where a high dose (100 nmol/L) of Ang-(1-12), Ang I and Ang II elicited a similar maximal contraction (Nagata et al., 2006), the maximal Ca^{2+} fluorescence induced by Ang-(1-12) was about 26% of the maximal response induced by Ang II in CHO cells. The divergent responses may be explained by the availability of appropriate converting enzymes in aorta preparations where Ang-(1-12) and Ang I is effectively converted to Ang II, leading to the same magnitude of vasoconstriction (Nagata et al., 2006). In the case of CHO cells transfected with AT_1R , Ang-(1-12) was not expected to be converted to Ang II because of a deficiency of appropriate metabolizing enzymes; hence, Ang II and Ang-(1-12) produced responses of different magnitude. A direct interaction with AT_1R may also explain the finding that Ang-(1-12) at a high concentration of 100 nmol/L, caused a small vasoconstriction that is suppressed by the AT_1R blocker CV-11974, but resistant to captopril (Nagata et al., 2006). Our interpretation is that at a higher concentration, Ang-(1-12) unmasks its direct action on AT_1R .

Similar to the Ang II response, the Ang-(1-12)-induced Ca^{2+} mobilization was dose dependently suppressed by losartan. With respect to CHO cells expressing the AT_2R , Ang II stimulated Ca^{2+} mobilization with an EC_{50} value of 9.68 nM; whereas, Ang-(1-12) failed to elicit a significant Ca^{2+} response even at a concentration as high as 5 μM , indicating that Ang-(1-12) interacts preferentially with AT_1R . This is an important pharmacological distinction between Ang-(1-12) and Ang II in that the latter acts on both AT_1R and AT_2R . A previous report has concluded that exogenous Ang-(1-12) acts on AT_1R within the nucleus of the solitary tract to modify the heart rate and arterial pressure of anesthetized rats, but probably via its metabolism to Ang II (Arnold et al., 2010).

In CHO cells transfected with AT_1R , Ang II stimulated ERK phosphorylation at a concentration as low as 1 nM; the effective concentration was about 300 nM for Ang-(1-12). Notwithstanding different potencies, Ang-(1-12) and Ang II stimulated phosphorylation of ERK, without a change of total ERK in CHO cells transfected with AT_1R , implying that these two peptides activate similar intracellular signaling pathways. Lastly, we validated the direct action of Ang-(1-12) on hypothalamic neurons expressing native AT_1R (Cato and Toney, 2005; Latchford and Ferguson, 2005). Ang II or Ang-(1-12) evoked a membrane depolarization that is sensitive to the blockade by losartan, but not by the AT_2R antagonist PD-123319, nor by the ACE inhibitor captopril. Taken together, our results in cell lines and hypothalamic neurons *in vitro* raise the possibility that Ang-(1-12) directly and preferentially activates AT_1R , albeit at a concentration higher than that of Ang II. Viewed in this context, Ang-(1-12) behaves like a partial agonist on AT_1R .

Ang II has a vital role in the regulation of cardiovascular activity and fluid electrolyte balance. In a G protein-dependent manner, Ang II mediates vasoconstriction and blood pressure modulation through a myriad of signaling pathways. Activation of AT₁R by Ang II leads to the stimulation of PLC β , PLD and PLA₂. Signals propagated along the PLC β pathway will eventually result in the contraction of smooth muscle cells in a Ca²⁺/calmodulin-dependent manner (Abraham et al., 1996). AT₁R is also involved in growth signaling through activation of PKC and mitogen-activated protein kinases, including ERK1/2, c-Jun N-terminal kinase and p38, which are implicated in vascular smooth muscle cell differentiation, proliferation, and migration (Kyaw et al., 2004; Shah et al., 2004). Ang II induces cellular growth and protein synthesis through regulation of PHAS-1 (inhibitor of eukaryotic initiation factor 4E) via activation of ERK (Rocic et al., 2001). In the present study we have characterized Ang-(1-12) in both Ca²⁺ mobilization and ERK phosphorylation assays. Both Ang-(1-12)-induced Ca²⁺ mobilization and ERK phosphorylation were abolished in the presence of the AT₁R selective antagonist losartan, indicating that these downstream signaling were mediated through AT₁R. The partial agonism of Ang-(1-12) in multiple signaling pathways suggested that the precursor peptide is a previously unrecognized signaling peptide on the AT₁R and may be implicated in vasoconstriction and cell growth in the cardiovascular system.

It is generally recognized that overactive Ang II is associated with many cardiovascular diseases, including inflammation, endothelial dysfunction, atherosclerosis, hypertension and congestive heart failure (Daugherty et al., 2004; Diep et al., 2002; Garg and Yusuf, 1995; Wassmann et al., 2004). Both AT₁R blocker and ACEI inhibitor are regarded as effective therapeutic agents in treating Ang II-related pathophysiological conditions. Although ACEI inhibitors are commonly used to reduce the conversion of angiotensinogen to bioactive Ang II, it has no effect on the production of Ang-(1-12), the synthesis of which is independent of the classic renin-angiotensin cascade (Trask et al., 2008). It is noteworthy that Ang-(1-12) is subsequently converted to Ang II by chymase, and treatment with ACEI inhibitors, therefore, may not control the formation of Ang II via Ang-(1-12) (Ahmad et al., 2011). Since the ACE-independent mechanisms could sustain the production of Ang I and Ang II from Ang-(1-12) despite treatment with ACEI inhibitors, angiotensin receptor antagonists which directly suppress Ang II-mediated effects through receptor blockade might represent a better alternative treatment than to ACE inhibition.

A physiological role of Ang-(1-12) in various tissues remains to be clarified. Our finding that Ang-(1-12) can directly activate AT₁R does not preclude its role as a precursor to the formation of Ang II, which serves as a signaling molecule in a variety of cells and tissues. It is possible that in the case of systemic bolus injection of Ang-(1-12), the latter is converted to Ang I and Ang II in the circulation where metabolizing enzymes are available (Nagata et al., 2006). With respect to the nervous system, immunohistochemical studies reveal the presence of Ang-(1-12) immunoreactive neurons and cell processes in the hypothalamic paraventricular nucleus (Chitravanshi et al., 2011), implying the peptide may be released from nerve endings and serve as an endogenous signaling ligand independent of Ang II. It is of interest to note that the concentration of Ang-(1-12) in the rat brain is 3- and 5-folds higher than that of Ang I and Ang II (Nagata et al., 2006). As a corollary, a fraction of endogenous Ang-(1-12) appears to be converted to Ang I and Ang II in the brain.

5. Conclusion

Ang-(1-12) mobilized Ca^{2+} and phosphorylated ERK in COS-7 and CHO cells transiently transfected with AT_1R cDNA, with an EC_{50} higher than that of Ang II. Ang-(1-12) responses were suppressed by the AT_1R antagonist losartan. Ang-(1-12) or Ang II depolarized hypothalamic neurons bearing native AT_1R in rat hypothalamic slices *in vitro*. Responses were suppressed by losartan, but not by the ACE inhibitor captopril. It may be concluded that, in addition to being a precursor to the formation of Ang I and Ang II, Ang-(1-12) may produce its biological activity by acting directly on AT_1R in a tissue-specific manner.

Acknowledgments

This work was supported in part by the Hong Kong RGC (HKUST 660107, 660108, 660109), UGC (T13-607/12R), and the Hong Kong Jockey Club, and by NIH Grants NS18710 and HL51314 from the National Institutes of Health. YHC was supported by NSC101-2918-I-039-005 from the National Science Council, Taiwan, and YZ by YunNan Provincial United Fund (No. 2012FB013).

Abbreviations

Ang-(1-12)	angiotensin-(1-12)
Ang I	angiotensin I
Ang II	angiotensin II
ACE	angiotensin converting enzyme
AT_1R	angiotensin receptor type I
AT_2R	angiotensin receptor type II
ERK	extracellular signal-regulated protein kinases
RUF	relative fluorescence units

References

- Abraham ST, Benscoter H, Schworer CM, Singer HA. In situ Ca^{2+} dependence for activation of Ca^{2+} /calmodulin-dependent protein kinase II in vascular smooth muscle cells. *J. Biol. Chem.* 1996; 271:2506–2513. [PubMed: 8576214]
- Ahmad S, Simmons T, Varagic J, Moniwa N, Chappell MC, Ferrario CM. Chymase-dependent generation of angiotensin II from angiotensin-(1-12) in human atrial tissue. *PLoS One.* 2011; 6(12):e28501. [PubMed: 22180785]
- Arnold AC, Isa K, Shaltout HA, Nautiyal M, Ferrario CM, Chappell MC, Diz DI. *Am. J. Physiol. Heart Circ. Physiol.* 2010; 299:H763–H771. [PubMed: 20562338]
- Cato MJ, Toney GM. Angiotensin II excites paraventricular nucleus neurons that innervate the rostral ventrolateral medulla: an in vitro patch-clamp study in brain slices. *J. Neurophysiol.* 2005; 93:403–413. [PubMed: 15356186]
- Chitravanshi VC, Proddutur A, Sapru HN. Cardiovascular actions of angiotensin-(1-12) in the hypothalamic paraventricular nucleus of the rat are mediated via angiotensin II. *Exp. Physiol.* 2011; 97:1001–1017. [PubMed: 22125313]
- Daugherty A, Rateri DL, Lu H, Inagami T, Cassis LA. Hypercholesterolemia stimulates angiotensin peptide synthesis and contributes to atherosclerosis through the AT_1A receptor. *Circ.* 2004; 110:3849–3857.

- Diep QN, Amiri F, Touyz RM, Cohn JS, Endemann D, Neves MF, Schiffrin EL. PPARalpha activator effects on Ang II-induced vascular oxidative stress and inflammation. *Hypertension*. 2002; 40:866–871. [PubMed: 12468571]
- Ferrario CM, Chappell MC, Tallant EA, Brosnihan KB, Diz DI. Counterregulatory actions of angiotensin-(1-7). *Hypertension*. 1997; 30:535–541. [PubMed: 9322978]
- Garg R, Yusuf S. Overview of randomized trials of angiotensin-converting enzyme inhibitors on mortality and morbidity in patients with heart failure. Collaborative Group on ACE Inhibitor Trials. *JAMA*. 1995; 273:1450–1456. [PubMed: 7654275]
- Isa K, Garcia-Espinosa MA, Arnold AC, Pirro NT, Tommasi EN, Ganten D, Chappell MC, Ferrario CM, Diz DI. Chronic immunoneutralization of brain angiotensin-(1-12) lowers blood pressure in transgenic (mRen2)27 hypertensive rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2009; 297:R111–R115. [PubMed: 19403863]
- Jessup JA, Trask AJ, Chappell MC, Nagata S, Kato J, Kitamura K, Ferrario CM. Localization of the novel angiotensin peptide, angiotensin-(1-12), in heart and kidney of hypertensive and normotensive rats. *Am. J. Physiol. Heart Circ. Physiol.* 2008; 294:H2614–H2618. [PubMed: 18408132]
- Kyaw M, Yoshizumi M, Tsuchiya K, Kagami S, Izawa Y, Fujita Y, Ali N, Kanematsu Y, Toida K, Ishimura K, Tamaki T. Src and Cas are essentially but differentially involved in angiotensin II-stimulated migration of vascular smooth muscle cells via extracellular signal-regulated kinase 1/2 and c-Jun NH2-terminal kinase activation. *Mol. Pharmacol.* 2004; 65:832–841. [PubMed: 15044612]
- Lankley CJ, Hodges JC, Klutchko SR, Himmelsbach RJ, Chucholowski AW, Connolly CJ, Neergaard SJ, Van Nieuwenhze M, Sebastian A, Quin J III, Essenburg AD, Cohen DM. Synthesis and structure-activity relationships of a novel series of non-peptide angiotensin II receptor binding inhibitors specific for the AT1 subtype. *J. Med. Chem.* 1991; 34:3248–3260. [PubMed: 1956044]
- Latchford KV, Ferguson AV. Angiotensin depolarizes parvocellular neurons in paraventricular nucleus through modulation of putative nonselective cationic and potassium conductances. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2005; 289:R52–R58. [PubMed: 15831764]
- Liu AMF, Ho MKC, Wong CSS, Chan JHP, Pau AHM, Wong YH. Gα_{16/z} chimeras efficiently link G_i-coupled receptors to calcium mobilization. *J. Biomol. Screen.* 2003; 8:39–49. [PubMed: 12854997]
- Mody SM, Ho MKC, Joshi SA, Wong YH. Incorporation of Gα_z-specific sequence at the carboxyl terminus increases the promiscuity of Gα₁₆ towards G_i-coupled receptors. *Mol. Pharmacol.* 2000; 57:13–23. [PubMed: 10617674]
- Nagata S, Kato J, Kuwasako K, Kitamura K. Plasma and tissue levels of proangiotensin-12 and components of the rennin-angiotensin system (RAS) following low- or high-salt feeding in rats. *Peptides*. 2010; 31:889–892. [PubMed: 20172005]
- Nagata S, Kato J, Sasaki K, Minamino N, Eto T, Kitamura K. Isolation and identification of proangiotensin-12, a possible component of the renin-angiotensin system. *Biochem. Biophys. Res. Commun.* 2006; 350:1026–1031. [PubMed: 17045572]
- New DC, Wong YH. Characterization of CHO cells stably expressing a Gα_{16/z} chimera for high-throughput assays. *Assay Drug Dev. Tech.* 2004; 2:269–280.
- Pereira HJ, Souza LL, Costa-Neto CM, Salgado MC, Oliveira EB. Carboxypeptidases A1 and A2 from the perfusate of rat mesenteric arterial bed differentially process angiotensin peptides. *Peptides*. 2012; 33:67–76. [PubMed: 22178042]
- Rocic P, Govindarajan G, Sabri A, Lucchesi PA. A role for PYK2 in regulation of ERK1/2 MAP kinases and PI 3-kinase by ANG II in vascular smooth muscle. *Am. J. Physiol. Cell Physiol.* 2001; 280:C90–C99. [PubMed: 11121380]
- Sasaki K, Yamano Y, Bardhan S, Iwai N, Murray JJ, Hasegawa M, Matsuda Y, Inagami T. Cloning and expression of a complementary DNA encoding a bovine adrenal angiotensin II type-1 receptor. *Nature*. 1991; 351:230–233. [PubMed: 2041569]
- 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. USA*. 2003; 100:8258–8263. [PubMed: 12829792]
- Shah BH, Yesilkaya A, Olivares-Reyes JA, Chen HD, Hunyady L, Catt KJ. Differential pathways of angiotensin II-induced extracellularly regulated kinase 1/2 phosphorylation in specific cell types: role of heparin-binding epidermal growth factor. *Mol. Endocrinol*. 2004; 18:2035–2048. [PubMed: 15143154]
- Stern JE, Galarreta M, Foehring RC, Hestrin S, Armstrong WE. Differences in the properties of ionotropic glutamate synaptic currents in oxytocin and vasopressin neuroendocrine neurons. *J. Neurosci*. 1999; 19:3367–3375. [PubMed: 10212296]
- Timmermans PB, Wong PC, Chiu AT, Herblin WF, Benfield P, Carini DJ, Lee RJ, Wexler RR, Saye JA, Smith RD. Angiotensin II receptors and angiotensin II receptor antagonists. *Pharmacol. Rev*. 1993; 45:205–251. [PubMed: 8372104]
- Trask AJ, Ferrario CM. Angiotensin-(1-7): pharmacology and new perspectives in cardiovascular treatments. *Cardiovas. Drug Rev*. 2007; 25:162–174.
- Trask AJ, Jessup JA, Chappell MC, Ferrario CM. Angiotensin-(1-12) is an alternate substrate for angiotensin peptide production in the heart. *Am. J. Physiol. Heart Circ. Physiol*. 2008; 294:H2242–2247. [PubMed: 18359898]
- von Bohlen und Halbach O. Angiotensin IV in the central nervous system. *Cell Tissue Res*. 2003; 311:1–9. [PubMed: 12483279]
- Wassmann S, Czech T, van Eickels M, Fleming I, Böhm M, Nickenig G. Inhibition of diet-induced atherosclerosis and endothelial dysfunction in apolipoprotein E/angiotensin II type 1A receptor double-knockout mice. *Circ*. 2004; 110:3062–3067.
- Westwood BM, Chappell MC. Divergent pathways for the angiotensin-(1-12) metabolism in the rat circulation and kidney. *Peptides*. 2012; 35:90–195.
- Zhang J, Pratt RE. The AT₂ receptor selectively associates with G_{iα2} and G_{iα3} in the rat fetus. *J. Biol. Chem*. 1996; 271:15026–15033. [PubMed: 8663053]

Highlights

- Ang-(1-12) or Ang II mobilizes Ca^{2+} in cell lines transfected with AT_1R cDNA
- Ang-(1-12) or Ang II induces ERK phosphorylation in cell lines
- Ang-(1-12) or Ang II depolarize neurons in rat hypothalamic slices
- Ang-(1-12) depolarization is suppressed by losartan and not affected by captopril
- Ang-(1-12) may directly act on AT_1R , in addition to being a precursor to the formation of Ang I and Ang II

Figure 1. Ang-(1-12) stimulated intracellular Ca^{2+} mobilization via the AT_1 receptor
 (A) CHO/16z25 cells transiently expressing the AT_1 or AT_2 receptors were labeled with Ca^{2+} sensitive fluorophore Fluo-4 and subjected to FLIPR assays with increasing concentrations of Ang II (1 pM to 100 nM) or Ang-(1-12) (5 pM to 5 μM). (B) Cos-7 cells transiently expressing the AT_1R were subjected to FLIPR assays as in (A). (C) CHO/16z25 cells expressing AT_1R were pre-incubated with increasing concentrations (1 nM – 10 μM) of losartan for 30 min prior to FLIPR assays with 5 μM of Ang-(1-12). Data were mean of peak fluorescence signals $\pm$ SEM of at least 3 different trials performed in triplicates, and normalized to the maximal response elicited by Ang II (as 100%) and the minimal response of vehicle-treated cells (as 0%). Dose-response curves were constructed by non-linear regression using GraphPad Prism 3.

Figure 2. Phosphorylation of ERK induced by Ang II and Ang-(1-12)

(A) CHO/16z25 cells transiently expressing AT₁R was serum-starved for 4 h before treatment with the indicated concentrations of Ang II or Ang-(1-12) for 5 min. (B) CHO/16z25 cells transiently expressing AT₁R was serum-starved and treated with 1 μ M Ang II or 10 μ M Ang-(1-12) for 5 min together with 10 μ M losartan. Total cell lysates were collected and the proteins were separated and electrotransferred for immunodetection of phosphorylated ERK (pERK) using an anti-phospho ERK antiserum. Total amount of ERK was also monitored as a loading control. Three individual trails yielded similar results as the representative blots shown in the figure.

Figure 3. AngII-induced depolarizations in rat hypothalamic neurons

A, a reproducible membrane depolarization was evoked by Ang II (1 mM) ejected from a micropipette positioned above the recording neuron as the interval between applications was kept at 30 min. B, Ang II-induced depolarization was not affected by pretreating the hypothalamic slice with captopril (10 μ M) for 30 min. C, Ang II-induced depolarization was suppressed by losartan (10 μ M).

Figure 4. Ang-(1-12)-induced depolarizations in rat hypothalamic neurons

A and B, Ang(1-12)-induced depolarization was not affected by captopril (10 μ M) nor by PD-123319 (10 μ M). C, Ang-(1-12)-induced depolarization was progressively suppressed 10 and 30 min following losartan (10 μ M) superfusion; the depolarization recovered to near control level following a 30 min wash with drug-free Krebs solution. D. histogram of Ang-(1-12) responses before and after captopril, PD-123319 and losartan treatment; Ang-(1-12) depolarizations were markedly suppressed by losartan; asterisk denotes statistically significant, $p < 0.05$. Numbers in min denote interval between two applications; responses were the average of 4-5 cells.