Cardioprotective Angiotensin-(1–7) Peptide Acts as a Natural-Biased Ligand at the Angiotensin II Type 1 Receptor

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Abstract—Hyperactivity of the renin–angiotensin–aldosterone system through the angiotensin II (Ang II)/Ang II type 1 receptor (AT1-R) axis constitutes a hallmark of hypertension. Recent findings indicate that only a subset of AT1-R signaling pathways is cardiodeleterious, and their selective inhibition by biased ligands promotes therapeutic benefit. To date, only synthetic biased ligands have been described, and whether natural renin–angiotensin–aldosterone system peptides exhibit functional selectivity at AT1-R remains unknown. In this study, we systematically determined efficacy and potency of Ang II, Ang III, Ang IV, and Ang-(1–7) in AT1-R–expressing HEK293T cells on the activation of cardiodeleterious G-proteins and cardioprotective β-arrestin2. Ang III and Ang IV fully activate similar G-proteins than Ang II, the prototypical AT1-R agonist, despite weaker potency of Ang IV. Interestingly, Ang-(1–7) that binds AT1-R fails to promote G-protein activation but behaves as a competitive antagonist for Ang II/Gi and Ang II/Gq pathways. Conversely, all renin–angiotensin–aldosterone system peptides act as agonists on the AT1-R/β-arrestin2 axis but display biased activities relative to Ang II as indicated by their differences in potency and AT1-R/β-arrestin2 intracellular routing. Importantly, we reveal Ang-(1–7) a known Mas receptor-specific ligand, as an AT1-R–biased agonist, selectively promoting β-arrestin activation while blocking the detrimental Ang II/AT1-R/Gq axis. This original pharmacological profile of Ang-(1–7) at AT1-R, similar to that of synthetic AT1-R–biased agonists, could, in part, contribute to its cardiovascular benefits. Accordingly, in vivo, Ang-(1–7) counteracts the phenylephrine-induced aorta contraction, which was blunted in AT1-R knockout mice. Collectively, these data suggest that Ang-(1–7) natural-biased agonism at AT1-R could fine-tune the physiology of the renin–angiotensin–aldosterone system. (Hypertension. 2016;68:1365-1374. DOI: 10.1161/HYPERTENSIONAHA.116.08118.) • Online Data Supplement

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The renin–angiotensin–aldosterone system (RAAS) constitutes a major endocrine system that regulates cardiovascular functions and has been the subject of extensive research both in physiology and pathophysiology. Several peptides are implicated in its regulation, all deriving from an enzymatic cascade with angiotensinogen as precursor and acting through highly diverse targets.

Among them, the octapeptide angiotensin II (Ang II or Ang-[1–8]), obtained after the conversion of angiotensinogen by renin and the subsequent action of the angiotensin-converting enzyme (ACE), has long been viewed as the main RAAS effector peptide. The effects of Ang II are essentially mediated through the angiotensin II type 1 receptor (AT1-R), a G-protein–coupled receptor (GPCR). Overstimulation of the ACE/Ang II/AT1-R axis has been assigned to physiopathological outcomes such as arterial hypertension, congestive heart failure, and chronic renal failure, principally by promoting excessive vasoconstriction, fibrosis, and cardiac or renal remodeling.1 Hence, the blockade of this axis, using specific ACE inhibitors or...
Ang II receptor blockers, has provided an effective therapeutic approach.2

Other bioactive Ang II–derived peptides obtained from amino or carboxyl cleavages of Ang II have been described, making the RAAS more complex than originally thought. Interestingly, despite their high amino acid sequence homology, most of these peptides display their own individual pharmacological and physiological properties. For instance, Ang-(1–7), which lacks the Ang II critical C-terminal phenylalanine residue as a result of ACE2-dependent cleavage, is described to exert vasodilatory and cardioprotective effects by acting predominantly through the Mas oncogene receptor (Mas-R).3 This Ang-(1–7) Mas-R axis currently seems to be an endogenous beneficial counter regulator of the Ang II/AT1-R axis and is subjected to downregulation in several cardiovascular diseases.4,5 More recently, Ang-(1–7) has also been shown to bind the cardioprotective angiotensin type II type 2 receptor (AT2-R),6 thus contributing, in part, to Ang-(1–7) actions, despite controversial discussions and recent discovery of PD-123,319, the major pharmacological AT2-R tool, as a nonspecific AT2-R antagonist.7–9 Finally, MrgD-R was also suggested as a new additional Ang-(1–7) receptor with physiologically vasorelaxant effects.9 Ang II can also directly lead to the production of angiotensin III (Ang III; Ang 2–8) by aminopeptidase N activity. Subsequent aminopeptidase N-mediated Ang III cleavage then generates angiotensin IV (Ang IV; Ang 3–8). However, the specific physiology of Ang III and Ang IV is poorly understood. Ang III was identified as a potent AT1-R and AT2-R agonist, displaying similar affinities and physiological effects as Ang II but was suggested to act predominantly in the central nervous system.10,11 However, Ang IV was long considered an inactive peptide but is now thought to act through the nonconventional insulin-regulated aminopeptidase receptor, regulating the vascular inflammatory response.11

The AT1-R has demonstrated versatile signaling functions12 basically classified in terms of G-protein–dependent and G-protein–independent signaling. The Gq/11 family is more often considered the prototypical pathway, despite the fact that Ang II/AT1-R can also mobilize the activation of the Gi/o and G12/13 G-protein families.13,14 Until now, Ang II/AT1-R/Gq axis overstimulation has been assigned to detrimental cardiovascular outcomes. However, AT1-R can also activate G-protein–independent pathways, mainly with β-arrestin (β-arr) as their core component15 and conversely associated with cardioprotective properties.15

Over the past decade, numerous studies have shown that different ligands for a given GPCR can induce or stabilize specific receptor conformations leading to the engagement of selective signaling pathways and referred as ligand-biased efficacy or ligand-directed trafficking.16 In this respect, AT1-R is perhaps one of the best templates, and several Ang II analog peptides have been identified as biased ligands. Among them, Sar1Ile4Ile8-angiotensin (SII) was initially described as a β-arr2–biased agonist unable to activate the Gq pathway.17,18 However; recent findings have revealed a higher mechanistic complexity of this biased compound that involves both β-arr and Gq effectors14–19 but leads to signaling pathways different from those promoted by Ang II.14 Interestingly, SII has demonstrated β-arr–dependent pharmacological properties, such as inotropic responses on isolated cardiomyocytes20; however, its low affinity for AT1-R has prevented further in vivo characterization of its biased activity.17 More recently, a drug discovery program conducted by Trevena Inc has led to the identification of SII-mimicking peptides with higher AT1-R affinity, which competitively antagonize the deleterious AT1-R/Gq pathway, whereas at the same time, it engaged the beneficial AT1-R/β-arr axis. Remarkably, these molecules elicit cardioprotective properties in vivo, reducing blood pressure (TRV120027) while acting as a mild inotropes (TRV120027 and TRV120023),21 and TRV120027 is currently in phase II clinical trials for the treatment of acute heart failure.22 It, thus, follows that drug selection of a subset of AT1-R signaling can accurately control the balance between cardioprotective and cardiodeleterious effects. Up to now, this concept has largely emerged from synthetic GPCR ligands and their use in pathophysiology, but it remains elusive whether endogenous ligands could underlie biased activity.

Despite the existence of numerous physiological Ang II–related peptides presenting high homology sequences (Table S2 in the online-only Data Supplement), no study has ever explored the endogenous biased activity of such peptides directly acting at AT1-R. In this study, we performed a systematic functional pharmacological characterization of 3 Ang II–related peptides, Ang III, Ang IV, and Ang-(1–7) and found biased activities of all peptides on the AT1-R/G protein and AT1-R/β-arr axis. For the first time, we reveal that the Mas-R–related agonist Ang-(1–7) antagonizes the Ang II/AT1-R/Gq pathway but promotes the recruitment of β-arr to the AT1-R, which could explain, in part, its cardioprotective properties.

Methods

An expanded Materials and Methods section is available in the online-only Data Supplement.

Reagents

Ang II, Ang III, Ang IV, and Ang-(1–7) were purchased from Sigma–Aldrich. Candesartan was kindly provided by J.L. Hansen. Coelenterazine 400a and h were purchased from Biotum.

Statistical Analysis

Statistical analysis and curve fitting were performed using Prism 5.01 (GraphPad Software, San Diego, CA). The statistical significance of the differences was assessed using appropriate statistical analysis as indicated in the different legends. Two-group comparisons were performed using paired or unpaired Student t tests after verification that values came from a Gaussian distribution. Multiple-group comparisons were conducted using 1-way ANOVA followed by a Dunnet test.

Results

Ang-(1–7) Is a Potent Antagonist of Ang II–Mediated G-Protein Activation at AT1-R

To evaluate the pharmacological profiling of Ang II–derived peptides at AT1-R, we first assessed the effects of Ang III, Ang IV, and Ang-(1–7) on AT1-R–mediated G-protein activation in AT1-R–expressing HEK293T cells using bioluminescence resonance energy transfer (BRET)–based probes measuring the interaction between the different Gt-RLuc8 probes and GFP10-Gq2 as previously described.14,23 To be
noticed, although this assay allows quantification/comparison of the different ligand-modulated BRET signals for a specific G-protein, the comparison of maximal ligand efficacy between different G-proteins is precluded.\textsuperscript{14,23} Results first indicated that among the peptides tested (Ang II, Ang III, Ang IV, and Ang-(1–7)) not one was able to activate the G-protein BRET probes in HEK293T cells in the absence of AT1-R overexpression, precluding the existence of endogenous signaling (Figure 1A; Figure S1). In contrast, when AT1-R-expressing cells were stimulated with 10 μmol/L RAAS peptides for 2 minutes, Ang II promoted a significant decrease in the BRET signal, indicative of G\textsubscript{q} and G\textsubscript{\beta\gamma} separation during the activation process, for all G\textsubscript{q} isoforms of the G\textsubscript{q}/o, G\textsubscript{q}/11, and G\textsubscript{o}13 families (Figure 1A; Figure S1), in accordance with the well-known predominant coupling of the AT1-R to these G-protein families.\textsuperscript{14} As expected, Ang III, known as an Ang II--mimicking peptide, activated similar G-proteins to that of Ang II and to the same extent (Figure 1A). More surprisingly, Ang IV also displayed full agonism at G-protein activation, similar to that of Ang II or Ang III (Figure 1A), although this peptide is not thought to bind to the AT1-R. In contrast to the other RAAS peptides, the Mas-R–related Ang-(1–7) presented no efficacy toward the different G-protein isoforms (Figure 1A; Figure S1). This effect could not rely on slower activation kinetics as no increase in the BRET response could be measured even after 10 minutes stimulation (Figure S2). Further characterization of the RAAS peptides on G\textsubscript{q} and G\textsubscript{i3} activation indicated significant differences in their potency (Figure 1B; Table S1). Ang II and Ang III stimulated G\textsubscript{q} and G\textsubscript{i3} with similarly high potencies, confirming Ang III as a potent AT1-R agonist, whereas Ang IV showed a lower potency for the activation of both the G-proteins. Interestingly, Ang IV also exhibited distinct potencies toward each G-protein isoform with an EC\textsubscript{50} < EC\textsubscript{50} < 10\textsuperscript{-9}M, indicative of a biased Ang IV activity relative to that of Ang II. Thus, Ang IV can be classified as a weak but full agonist at the AT1-R. Again, we confirmed that no G-protein activation could be detected in the presence of Ang-(1–7) even over a large range of concentrations. The potencies measured for the 4 RAAS peptides on G-protein activation profiles were similar on further downstream G-protein–dependent Ca\textsuperscript{2+} signaling (Figure S3). Although these results could indicate that Ang-(1–7) does not bind to the AT1-R as expected, it could also point to a selective lack of efficacy at the AT1-R/G protein axis. To get further insight into these possibilities, we performed direct competition binding studies on membranes from AT1-R–expressing HEK293T cells using native \textsuperscript{125}I-Ang II. Interestingly, all 4 RAAS peptides competed for Ang II binding (Figure 2), confirming their direct action on AT1-R. In agreement with the G-protein pharmacology, Ang II and Ang III displaced \textsuperscript{125}I-Ang II binding with identical high nanomolar affinities, whereas Ang IV presented a considerably lower affinity in the micromolar range. More surprisingly, Ang-(1–7) specifically bound AT1-R because it completely competed for \textsuperscript{125}I-Ang II binding with a Ki of 360 nmol/L. Similar affinity was measured in cells expressing the G-protein BRET probes (Figure S4), precluding a potential impact of G-protein overexpression on Ang-(1–7)–binding properties.

Given the lack of efficacy of Ang-(1–7) on the AT1-R/G-protein pathways but its ability to bind AT1-R, we tested the functional antagonistic potency of this peptide on Ang II–induced G\textsubscript{q} and G\textsubscript{i3} activation. As indicated in Figure 3 (top), Ang-(1–7) dose-dependently shifted the EC\textsubscript{50} of the Ang II concentration response curve to the right with no modification of the maximal efficacy for both the G proteins. This antagonist efficacy of Ang-(1–7) was constant independently of cell surface AT1-R low or high expression (Figure S5), precluding the contribution of spare receptors and was confirmed in G\textsubscript{q}-dependent Ca\textsuperscript{2+} signaling (Figure S6). Schild analysis was consistent with Ang-(1–7) being a competitive antagonist with slope values of linear regression close to the unit (Figure 3, bottom). Interestingly, the relative antagonistic potency of Ang-(1–7) was significantly better for G\textsubscript{i3} activation than for G\textsubscript{q}, suggesting that the nature of the G\textsubscript{q} subunit influences Ang-(1–7) antagonist properties. Altogether, these results clearly indicate that Ang-(1–7) acts as a natural neutral antagonist at AT1-R–dependent G-protein signaling.

Ang-(1–7) Is a Weak Agonist on AT1-R–Dependent β-arrr2 Activation

We next tested the efficiencies of the RAAS peptides on β-arrr2 activation mediated by the AT1-R. First, we evaluated β-arrr2 recruitment to the AT1-R by measuring the BRET signal between AT1-R-Venus and RLuc-β-arrr2 in living HEK293T cells. Contrary to that observed in the G-protein assay, all 4 RAAS peptides promoted a time-dependent β-arrr2 recruitment to AT1-R (Figure 4A). It should be noted that the kinetic curves fitted significantly better a 2-component model for Ang II (F=11) and Ang III (F=43), whereas a 1-component model was better suited to Ang IV (F=1) and Ang-(1–7) (F=not applicable; Table S3), suggesting that Ang II and Ang III recognize 2 distinct AT1-R states that could be related to different receptor conformations/phosphorylations as previously described,\textsuperscript{24} whereas only one is promoted by Ang IV and Ang-(1–7). Ang II and Ang III also exhibited similar recruitment kinetics, whereas both Ang IV and Ang-(1–7) recruited β-arrr2 at a slower rate and at lower maximal levels (80% and 76% of Ang II maximal recruitment, respectively; Table S3). Taken as a whole, maximal BRET-β-arrr2 recruitments could quantitatively imply Ang IV/Ang-(1–7) less efficient than Ang II/ Ang III. However, as we previously demonstrated it,\textsuperscript{24} because of the complexity of the RET restrictions, maximal BRET recruitments could also be indicative of conformational rearrangements within the receptor/β-arrr2 complex. In agreement, when swapping the energy donor and acceptor (modification of dipole orientation) on the β-arrr2 probe and measuring the interaction between AT1-R-Venus but another C-terminal β-arrr2-RLuc probe, the maximal BRET observed for Ang IV was now similar to that of Ang II or Ang III (92% and 102%, respectively), whereas Ang-(1–7) response was unchanged (Figure 4B; Table S3). Altogether, these results argue for Ang II/Ang III, Ang IV, and Ang-(1–7) stabilizing at least 3 different states of the AT1-R/β-arrr2 complex. Of note, Ang II/Ang III and Ang IV/Ang-(1–7) kinetics still better fitted, respectively, a 2- and a 2-component model (Table S3). Dose–response experiments indicated that Ang II and Ang III presented a better potency for
Figure 1. Pharmacological characterization of renin–angiotensin–aldosterone system (RAAS) peptides on angiotensin II (Ang II) type 1 receptor (AT1-R)–mediated G-protein activation using bioluminescence resonance energy transfer (BRET) assay. HEK293T cells coexpressing the indicated Gα-iFLuc8 isoforms, along with GFP10-Gγ2 and Gβ1 in the presence or absence of HA-AT1-R (cartoon diagram) were stimulated or not (vehicle) with 10 μmol/L RAAS peptides (A) or increasing ligand concentrations (B) for 2 min. Results are expressed as the difference in the BRET signal measured in the presence or absence of ligand (A and B) and are normalized according to the percentage of maximal Ang II response (B). Data represent the mean±SEM of 3 to 8 independent experiments. Statistical significance of each ligand stimulation between HA-AT1-R expressing and nonexpressing cells was assessed using an unpaired Student t test, (*P<0.05) or 1-way ANOVA followed by a Dunn test for comparison between ligands. n.s. indicates not significant.
β-arr2 recruitment, whereas Ang IV and Ang-(1–7) behaved as weaker agonists (Figure 4C; Tables S1 and S3). Similar behaviors were measured whatever the AT1-R expression levels (Figure S7), thus, again arguing against spare receptors involvement. It should be noted that the EC50 of Ang-(1–7) on β-arr2 recruitment assay (Table S1) is closely related to its binding affinity (Figure 2). Moreover, candesartan, an AT1-R selective antagonist, inhibited both Ang II and Ang-(1–7) dose-dependent β-arr recruitment to AT1-R (Figure S8), confirming Ang-(1–7) as a specific AT1-R agonist on the β-arr pathway.

These results were confirmed on the AT1-R/β-arr2 complex internalization profile visualized by using confocal fluorescence microscopy in HEK293T cells coexpressing AT1-R-Venus and β-arr2-Cherry (Figure S9) and specific of overexpressed AT1-R (Figure S10), confirming Ang-(1–7) as a specific AT1-R agonist on the β-arr pathway.

Ang-(1–7) attenuates phenylephrine-induced contraction of abdominal aorta via AT1-R

We finally aimed to identify the physiological relevance of an AT1-R/Ang-(1–7) axis. Because blood vessels are an important site of production/actions of both Ang II and Ang-(1–7),5 we examined the effect of Ang-(1–7) on the phenylephrine-induced contraction of abdominal aorta, demonstrating higher AT1-R responses,25 in wild-type or AT1-R knockout mice. Abdominal aorta from AT1-R knockout mice did not exhibit alteration in gene expression of the two other Ang-(1–7) receptors, Mas-R or AT2-R (Figure S11). In wild-type mice, 1 or 0.1 µmol/L Ang-(1–7) dose-dependently decreased by ≤20% the phenylephrine-induced contraction, whereas this effect was totally blunted in AT1-R knockout mice (Figure 5A) or in the presence of the AT1-R–specific antagonist candesartan (Figure 5B). In contrast, the Mas-R (A-779) or the AT2-R (PD 123,319)–specific antagonists had no effect (Figure 5B), demonstrating the specificity of Ang-(1–7) action through endogenously expressed AT1-R. Interestingly, when similar experiences were conducted but using Ang II as vasoconstrictor, we found that Ang-(1–7) conversely potentiated the Ang II–promoted contraction, which was totally inhibited by the specific Mas-R antagonist A-779, then suggesting an action through the Mas-R (Figure S12). Hence, these results reinforce the notion that Ang-(1–7) physiological actions rely on a complex interplay between all receptors of the RAAS, with some of them that could potentially rely on the AT1-R.

Discussion

This study provides for the first time a benchmark reference for the relative potencies and efficacies of all the major endogenous RAAS peptides derived from Ang II for the heterotrimeric G-protein activation and the β-arr recruitment through the AT1-R. Strikingly, our results reveal a biased activity of Ang III, Ang IV, and Ang-(1–7) at the AT1-R when compared with the reference agonist Ang II for all 3 signaling pathways analyzed in this study (Gαq, Gαi3, and β-arr2).
A major finding of our study is that Ang-(1–7), which is thought to be a specific predominant Mas-R–specific agonist ligand\(^3\)\(^4\) with very different physiological but sometimes conflictual effects,\(^5\) behaves also as a natural competitive neutral antagonist for AT1-R on the G-protein–dependent signaling while simultaneously acting as an agonist for \(\beta\)-arr–related signaling (Figure 6). Given the cardiodeleterious character of the AT1-R/Gq axis and the opposite cardioprotective benefit of the AT1-R/\(\beta\)-arr axis, this finding brings to the fore the components underlying the physiological protective signaling associated with Ang-(1–7). In this sense, the Ang-(1–7)/AT1-R RAAS component could be considered the physiological natural side of the TRV120027/AT1-R axis currently targeted for the efficient treatment of acute heart failure.

Decreased expression of ACE2 (Ang-[1–7]–forming enzyme) and its associated Ang-(1–7) product with consequent loss of cardioprotective Ang-(1–7)/AT1-R axis is a general hallmark of cardiovascular diseases such as heart failure\(^4\)\(^5\) that could be restored by TRV120027 treatment. Indeed, the cardioprotective benefits of Ang-(1–7) most probably arise from its GPCR pluridimensional actions, at least through the Mas-R, as suggested by some studies through the AT2-R, but also the AT1-R. This notion is further emphasized by the recent discovery of MrgD as another novel receptor for Ang-(1–7) regulating the physiological arterial pressure.\(^9\)

Physiological relevance of the Ang-(1–7)/AT1-R axis could be, however, questioned given that the affinity/potency of Ang-(1–7) for AT1-R is modest (≈300 nmol/L) and relatively far from plasma concentrations generally described, with significant differences in between rodents (nmol/L)\(^26\)\(^27\) or human species (pM).\(^28\)\(^–\)\(^30\) Nevertheless, despite the RAAS was originally described as a circulating system, it is now widely accepted that a distinct local RAAS does exist in several tissues,\(^31\) thus promoting a close ligand-receptor proximity to ensure an accurate spatiotemporal regulation of RAAS actions similar to that observed for neurotransmitters concentrations in the synaptic cleft after release.\(^32\) In line with this assumption, Ang II levels were quantified >100-fold higher in interstitial fluid of the heart than plasma levels.\(^33\) In such local RAAS context, Ang-(1–7) could, thus, reach sufficient concentrations in tissues to regulate AT1-R activity. Ang-(1–7) could, thus, be found to reach 30 pmol/g in renal aorta of the Wistar Kyoto rat\(^34\) and ≤200 pmol/g in mice kidney tissue or 300 pmol/g in diabetic mice,\(^35\) a concentration comparable to the Ki of Ang-(1–7) for AT1-R in our study. Now, even in a local environment, given the higher affinity of Ang II for AT1-R compared with Ang-(1–7), the probability to favor the Ang-(1–7)/AT1-R axis over that of Ang II/AT1-R axis will rely essentially on higher Ang-(1–7) concentrations than Ang II.

Figure 4 Continued. receptor (AT1-R)–mediated \(\beta\)-arr2 recruitment). Real-time measurement of BRET signals in HEK293T cells coexpressing HA-AT1-R-Venus and \(\beta\)-arr2-RLuc (A) or RLuc-\(\beta\)-arr2 (B), as illustrated in the cartoon diagrams, in the presence or absence (vehicle) of 10 μmol/L Ang II, Ang III, Ang IV, or Ang-(1–7) (A and B) or increasing concentrations of the indicated ligands (C). Results are expressed as the difference in the BRET signal measured in the presence or absence of ligand (A and B) or as the percentage of the maximal BRET signal measured in the presence of Ang II (C). Data represent the mean ± SEM of 9 to 5 independent experiments.
or high expression level of AT1-R. In that sense, Ang-(1–7) concentrations will depend on the expression level/activity of ACE2 in each tissue, and several strategies relying on increasing ACE2 expression have been shown to effectively counteract the Ang II detrimental effects.36,37 Conversely, tissues exhibiting high level of AT1-R expression that colocalize with ACE2, like the healthy kidney,38 could also ensure specific Ang-(1–7)/AT1-R–dependent physiological functions.

Despite it never having been thoroughly evaluated, data from several reports are in line with the concept that Ang-(1–7) could target the AT1-R. Indeed, Ang-(1–7) only differs from Ang II in the lack of C-terminal phenylalanine residue (Phe8; Table S2). Previous structure/function studies using Ang II analog peptides have already highlighted Phe8 as a key determinant in directing the activation of AT1-R/G-protein–dependent signaling and more recently in the β-arrest functional selectivity.39

Also, many studies have investigated the potential competitive binding between Ang-(1–7) and Ang II for the AT1-R but with contrasting results. Although some authors have described Ang-(1–7) as a high affinity competitor of Ang II,40 others have reported competition but with low affinity41,42 or even a lack of competition.6 All these binding controversies most probably rely on the use of different tissues preparations exhibiting different RAAS receptors expression levels that could likely influence Ang-(1–7)–binding affinity given its multidimensional action on different RAAS receptors (Mas-R, AT1-R, AT2-R…). The use of modified Ang II radiotracer6 could also have affected Ang-(1–7)–binding properties. In this study, we clearly demonstrated that Ang-(1–7) can fully compete for native Ang II binding to AT1-R. Moreover, downregulation of cell surface AT1-R by high concentrations of Ang-(1–7) has been observed in heterologous and endogenous systems.41,43

Finally, we showed that some of the Ang-(1–7) physiological effects could rely, in part, on its interaction with AT1-R because Ang-(1–7) could attenuate phenylephrine-induced aorta contraction, an effect lost in knockout–AT1-R mice. Thus, despite Ang-(1–7)/Mas-R axis was identified as a major cardioprotective axis in many cardiovascular diseases with

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**Figure 5.** Effect of angiotensin-(1–7) (Ang-[1–7]) on phenylephrine (PE)-induced contraction of abdominal aorta. **A**, Abdominal aorta contraction was promoted by the incubation of aorta rings from wild-type (WT) or knockout (KO)-Ang II type 1 receptor (AT1-R) mice with different concentrations of PE during 30 minutes in the presence or not (control-vehicle) of 1 µmol/L Ang-(1–7). Data are expressed as the percentage of maximal PE-contractile response obtained in controls and represent the mean±SEM of 5 to 8 independent experiments (5–8 mice). Statistical significance was assessed using 2-way ANOVA followed by Bonferroni test (*P<0.05 compared with WT control, **P<0.01, and ***P<0.001 compared with WT Ang-[1–7]; inset). Abdominal aorta contraction was promoted by the incubation of aorta rings from WT mice with 10^{-4} mol/L PE during 30 min in the presence or not (−) of 100 nmol/L or 1 µmol/L Ang-(1–7). Data are expressed as the percentage of the 10^{-4} mol/L PE-contractile response and represent the mean±SEM of 7 to 15 independent experiments. Statistical significance was assessed using 1-way ANOVA followed by a Dunnett multiple comparison test (*P<0.05, **P<0.01). **B**, Abdominal aorta contraction was promoted by the incubation of aorta rings from WT mice with 10^{-4} mol/L PE during 30 min in the presence or not (−) of 1 µmol/L Ang-(1–7) alone or in combination with 10 µmol/L AT1-R (Candesartan) or AT2-R (PD-123, 319), or Mas oncogene receptor (A-779) selective antagonists. Data are expressed as the percentage of 10^{-4} mol/L PE-contractile response obtained in controls (−) and represent the mean±SEM of 7 to 15 independent experiments (5–8 mice). Statistical significance was assessed using 1-way ANOVA followed by a Dunnett multiple comparison test (*P<0.05, ***P<0.001, compared with Ang-(1–7)–treated aorta). n.s. indicates non significant.

**Figure 6.** Schematic representation of potential physiological beneficial angiotensin-(1–7) (Ang-[1–7]) actions on Ang II type 1 receptor (AT1-R). Ang-(1–7) acts as an antagonist on Ang II/AT1-R–mediating Gq activation, known to be a cardiodeleterious pathway but also on Ang II/AT1-R–mediating Gi3 activation whose physiological role is unknown. Concomitantly, Ang-(1–7) behaves as a direct agonist on AT1-R/β-arrestin 2 pathway with cardioprotective properties.
also the more recent but controversial Ang-(1–7)/AT2-R axis, Ang-(1–7)/AT1-R could represent a new additional protective axis as well. Indeed, molecular mechanisms underlying Ang-(1–7)–related cardioprotection are not clearly characterized and remain poorly understood. Its action through the Mas-R was predominantly highlighted, in part, because of the lack of other known Ang-(1–7) targets. However, Ang-(1–7) physiology is controversial and not systematically related to cardioprotection. For instance, in experimental hypertension and according to the model, Ang-(1–7) could behave as well as cardioprotective by reducing hypertension or as cardiodeleterious by conversely potentiating hypertension.44 These discrepancies are in agreement with our results showing that Ang-(1–7) decreases phenylephrine-induced contraction through the AT1-R, whereas conversely in the same experimental model, it potentiates Ang II–induced abdominal aorta contraction through the Mas-R. Overall, these results likely argue for the pluridimensional actions of Ang-(1–7). Depending on the concentrations of peptide and receptors in the different cells of a tissue, 1 Ang-(1–7)/receptor axis will be favored compared with the other one, the physiological relevance of which will have to be identified in the future.

A second new finding of our study is that Ang IV demonstrates biased activity for all signaling pathways (Gq,Gs, Gβγ, and Gαi3/αi5) at the AT1-R, displaying different potencies for each of them (EC50 ααα<s>ααα</s>EC50 αi3<EC50 αi5 β-arr2; Table S1), whereas the potency of Ang II was similar for the different signals. Despite accumulating evidence of Ang IV action through insulin-regulated aminopeptidase, our results also support the notion that several Ang IV physiological effects could be mediated through AT1-R.1,13,46 Hence, depending on its concentration in physiological or pathophysiological situations, Ang IV can act as an efficient AT1-R agonist. Of note, Ang IV, which lacks the first 2 N-terminal amino acids of Ang II (Table S2), is sufficient for producing full agonism on both G-protein and β-arr recruitment, in line with previous structure/function studies.47 Finally, we have shown that Ang II and Ang III, which differ in only the first N-terminal amino acid (Asp’), exhibit similar agonist efficacies and potencies at the AT1-R, for both G-protein activation and β-arr recruitment, in line with our binding data showing a closely related high affinity of the AT1-R for Ang II and Ang III.48 Hence, Ang III acts as an Ang II–like endogenous AT1-R agonist. It is, thus, not surprising that both peptides were shown to be equipotent pressor agents.48 Despite the effect being modest, we found that Ang II and Ang III displayed slightly different rank orders of potency for β-arr2 compared with G-protein signaling (Table S1), making Ang III more likely to be a G-protein–biased agonist, and suggesting the stabilization of distinct receptor states. Despite their similar pharmacology, differential tissue-specific activities of the Ang II– and Ang III–producing enzymes could support the predominant action of one peptide over the other. In this sense, Ang III is suggested as the lead effector of the central RAAS in the control of heart failure.10

In conclusion, beside the classical Ang II/AT1-R pathway, our present work presents strong in vitro pharmacological evidence for the existence of additional Ang III, Ang IV, and Ang-(1–7) actions directly at AT1-R. Thus, this study paves the way for future investigations to identify the specific in vivo relevance of such Ang II peptides/AT1-R axis. In this context, the present in vivo demonstration of the Ang-(1–7)/AT1-R axis counteracting phenylephrine-induced abdominal aorta contraction highlights Ang-(1–7)/AT1-R as a potential therapeutic target in hypertension.

**Perspective**

This study is the first demonstration of biased activity of endogenous Ang II–related peptides at the AT1-R, leading to the description of new RAAS peptides/AT1-R axis with highly specific pharmacology. These results open the door for future investigations to delineate the physiological relevance of the natural-biased activity of these peptides and may potentially lead to a therapeutic value. In this context, our study suggests that the cardioprotective role generally assigned to Ang-(1–7) through the Mas-R more likely relies on intricate and pluridimensional receptor actions that could, in part, involve its biased activity at the AT1-R. Despite an exhaustive literature on biased agonism at GPCRs, few examples of natural-biased ligands have been described.49-51 Our results, by providing not only the existence of natural agonists but also the natural antagonists/biased agonists, unveil that functional selectivity of endogenous GPCR ligands could, thus, constitute a more general evolutionary process allowing an accurate fine-tuning of the physiological receptor response. Thus, better delineation of physiological-biased activity of the RAAS peptides at the different RAAS receptors should certainly help refining drugs for hypertension management and allow enhancing desirable efficacies while limiting the undesirable ones.

**Acknowledgments**

We thank the Genotoul Imagerie–Toulouse Réseau Imagerie platform at the I2MC for confocal imaging experiments, Marie Caquiel for help in quantitative PCR experiments, Céline Mias, Maxime Branchereau, and Christophe Heymes for helpful discussions, and Dr Heverker for critical reading of the article.

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**Disclosures**

None.

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**Novelty and Significance**

**What Is New?**

- Angiotensin III (Ang III), Ang IV, and Ang-(1–7) exhibit biased activity at Ang II type 1 receptor (AT1-R) receptor.
- Ang-(1–7) acts as an agonist on AT1-R/β-arrestin pathway and an antagonist on the Ang II/AT1-R/G-protein pathway.
- Ang-(1–7) blunts phynylephrine-induced aorta contraction through an AT1-R-dependent mechanism.

**What Is Relevant?**

- Ligand-biased activity of synthetic ligands has been recently demonstrated at AT1-R and some are in development for human cardioprotection, but nothing is known about physiological existence of such biased activity of renin–angiotensin–aldosterone system (RAAS) peptides at the AT1-R.
- This study provides new insights into the physiological RAAS regulation by demonstrating that biased activity should be considered as a novel way to fine-tune the RAAS. It, thus, opens new opportunities to target more selectively the RAAS and to increase the desirable efficacies of drugs in cardiac diseases.

**Summary**

The main finding of this study is that RAAS natural peptides exhibit biased activities at the AT1-R. Particularly, Ang-(1–7) a cardioprotective peptide commonly considered to essentially act through the Mas oncogene receptor, promotes selective activation of the cardioprotective AT1-R/β-arrestin while antagonizing the detrimental Ang II/AT1-R/Gq axis and attenuates phynylephrine-induced aorta contraction through an AT1-R mechanism.
Cardioprotective Angiotensin-(1–7) Peptide Acts as a Natural-Biased Ligand at the Angiotensin II Type 1 Receptor
Ségolène Galandrin, Colette Denis, Cédric Boularan, Jacky Marie, Céline M’Kadmi, Claire Pilette, Caroline Dubroca, Yvan Nicaise, Marie-Hélène Séguelas, Du N’Guyen, Jean-Louis Banères, Atul Pathak, Jean-Michel Sénard and Céline Galés

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The cardioprotective angiotensin 1-7 peptide acts as a natural biased ligand at the angiotensin II type 1 receptor


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° Département d'histopathologie, Centre Hospitalier Universitaire de Toulouse, F-31059 Toulouse, France. † Service de Pharmacologie Clinique, Faculté de médecine, Centre Hospitalier Universitaire de Toulouse, F-31432 Toulouse, France.
Supplemental Materials and Methods

**cDNA expression vectors**
Plasmids encoding human G protein subunits Gαi1-91-RLuc, Gαi2-91RLuc, Gαi3-91-RLuc, GαoA-91-RLuc, GαoB-91-RLuc, Gα11-98-RLuc, Gaq-97-RLuc, Gαs-113-RLuc, Gα12-115-RLuc, Gα13-106-RLuc, Gβ1, GFP10-Gγ2, rat AT1-R-Venus, RLuc-βarrestin2, βarrestin2-RLuc and βarrestin2-Cherry were constructed as previously described\(^1\). The vector encoding rat HA-AT1-R was a generous gift from J.L. Hansen (Novo Nordisk, Måløv, Denmark).

**Cell culture and transfection**
Human embryonic kidney 293 cells (HEK293T) cells were cultured in DMEM Glutamax supplemented with 10 % (v/v) FBS and 100 units/ml penicillin/streptomycin at 37°C in a humidified atmosphere at 5 % CO₂. Transient transfections were performed 24 h after cell seeding using polyethylenimine (PEI, Polysciences Inc.) or X-tremeGENE 9 DNA Transfection Reagent (Roche, Mannheim, Germany), according to the manufacturer’s protocol.

**Radio-iodination of Angiotensin II**
The angiotensin peptide was dissolved in 0.5 M PBS pH 7.5 at a concentration of 1 mM. 10µL (37 MBq, 1 mCi) of \[^{125}\text{I}\]Na (PerkinElmer) was added to 10 µL of the peptide solution. The reaction was initiated by adding 10 µL of a freshly prepared chloramine T solution (1 mg/ml in 0.5 M PBS pH 7.5). The mixture was allowed to react at room temperature for 2 min with intermittent stirring. The reaction was stopped by addition of 400 µL of a freshly prepared Na₂S₂O₅ solution (2 mg/ml in 0.5M PBS pH 7.5). The mono-iodinated angiotensin II was separated from the unreacted radioiodine and the unlabelled peptide with an HPLC-reverse phase system on a C18 column. The peptides were eluted from the column with 0.1 % trifluor-acetic acid (TFA) and 10 % acetonitril for 5 min followed by a linear acetonitril gradient of 10 to 50 %.

**Competition binding on the AT1-R**
Competition binding experiments were performed on crude plasma membranes of HEK293T cells transiently expressing the HA-AT1-R. Plasma membranes (15 µg) were incubated in a 90 µL solution of 50 mM Tris/HCl pH 7.2, 5 mM MgCl₂, 1 mg/ml BSA containing \[^{125}\text{I}\]-Angiotensin II (3 nM final concentration) and varying concentrations of cold ligand. The solution was incubated for 30 min at 30°C and bound ligand was separated from free by vacuum filtration through GF/C filters pretreated for 1h with 0.5% polyethylenimine. Radioactive bound ligand was estimated by liquid scintillation spectrometry. pKi/Ki values were obtained from competition curves analysis with GraphPad Prism v5 software.

**Bioluminescence Resonance Energy Transfer (BRET) measurements**
BRET experiments were performed as previously described\(^4\). Receptor and G protein- or β-arrestin-encoding constructs were transiently cotransfected into HEK293T cells as indicated in the figure legends. Forty-eight hours after transfection, cells were washed with PBS, detached in PBS / 5 mM EDTA and resuspended in PBS / 0.1% (w/v) glucose at room temperature. Gprotein activation experiments were performed using BRET2. For this purpose, cells were distributed (80 µg of protein per well) in a 96-well microplate (Wallac, PerkinElmer Life and Analytical Sciences) and incubated in the presence of the different ligands for 2 min. BRET2 between RLuc8 and GFP10 was measured immediately after the
addition of the RLuc substrate coelenterazine 400a (5 μM, Biotum). In contrast, β-arrestin recruitment was assessed using BRET1. Cells were distributed (80 μg of protein per well) in a 96-well microplate, incubated with 5 μM coelenterazine h for 7 min, then stimulated with ligands. For kinetics studies, BRET1 between RLuc and Venus was measured immediately after ligand injection and collected at 20 s intervals for 15 min. For β-arr2 dose response curves, BRET was measured 15 min after ligand addition. Readings were collected using a modified Infinite F500 (Tecan Group Ltd) as previously described²⁴. The BRET signal was calculated by the ratio of GFP10 (510-540 nm) to RLuc8 (370-450 nm) for BRET2 readings or Venus (520-570 nm) to RLuc (370-480 nm) for BRET1 readings.

**Intracellular Calcium Mobilization Assay.**
Calcium production was measured in HEK293T cells stably expressing mitochondria-targeted apoaequorin and transiently transfected with HA-AT1-R. The assay is based on luminescence emission of mitochondrial aequorin following intracellular Ca²⁺ release and was performed as described in {Sauliere, 2012 #10}.

**Immunofluorescence confocal microscopy**
For immunofluorescence, HEK293T cells were transiently transfected using X-TremeGENE 9 DNA in 6-well plates containing glass coverslips precoated with poly-L-Lysine (Sigma-Aldrich). Forty-eight hours after transfection, cells were serum-starved for 2 h then stimulated with the different ligands. Reactions were stopped on ice and cells were fixed for 20 min in 4 % paraformaldehyde, then washed with PBS and mounted with Mowiol containing DAPI. Confocal images were acquired using a LSM 780 microscope, piloted by manufacturer software, with a × 63 Plan-Neofluar objective (Carl Zeiss). Colocalization between proteins (correlation coefficient) was analyzed using the Manders co-efficient⁵ using ZEN software.

**Quantification of Cell Surface Receptors by ELISA**
Twenty-four hours post-transfection of HEK293T cells with pcDNA3.1(+) (control) or different levels of vector encoding HA-AT1-R (for G-protein activation) or HA-AT1-R-Venus (for βarr2 recruitment) in the presence of constant levels of vectors encoding either the heterotrimeric G protein subunits (Gαq-RLuc8 or Gαi3-RLuc8, GFP10-Gγ2 and Gβ1) or encoding RLuc-βarr2, cells were split into 24-well plates (300,000 cells/well). Twenty-four hours later, cells were fixed in 4 % paraformaldehyde, saturated with PBS/1 % BSA, and incubated with the primary anti-HA antibody (clone 16B12, Covance) and then with HRP-labeled secondary antibody (Sigma) After washing, the immunoreactivity was revealed by the addition of the HRP substrate 3,3′,5,5′-Tetramethylbenzidine (BD Bioscience San Diego, CA) according to manufacturer's instructions and the plates were read at 450 nm in a microplate reader (Varioskan Flash, Thermo Electron). OD obtained for control condition (background) was subtracted from each OD obtained with cells expressing different HA-AT1-R expression levels.

**Total RNA isolation and real-time quantitative RT-PCR**
Total RNA extraction from abdominal aorta was performed using Trizol according to the manufacturer’s instructions (TRI Reagent®, Molecular Research Center). First-strand cDNA was synthesized using the superscript II RT-PCR system (Invitrogen) with random hexamers. Negative controls without reverse transcriptase were conducted to control for the absence of genomic DNA contamination. Thirteen nanograms of cDNA from RT reaction were then mixed with specific primers for mouse AT2-R (Forward 5′GCAGTGGAGGAGCTCGGAAC3′; Reverse 5′CTCAGCTCCGGCATGCACCTCTTA3′) or Mas-R (Forward 5′GCGGCGTCATCATCTTCACTAG3′; Reverse
5’CCACGTGTCTTCCGTATCTT3’) and EVA green mix (Euromedex). Real-time PCR was performed in 96-well plates using an ABI StepOne (Applied Biosystems). Geometric mean values of GAPDH housekeeping gene was used for normalization. Melting curve analysis was performed to ensure a single PCR product and a specific amplification. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Vascular reactivity studies
KO-AT1-R mice (B6.129P2-Agtr1atm1Unc/J) were purchased from the Jackson Laboratory while wild-type and (WT) C57/Bl6 mice were purchased from Harlan laboratories. All studies were performed on male and age-matched mice. Experimental animal protocols were carried out in accordance with the French regulation guidelines for animal experimentation. After anesthesia with pentobarbital sodium (50 mg/kg, ip) of WT and KO mice, the abdominal aorta was excised, cleaned of fat and connective tissue and cut into rings (2 mm in length). Aortic segments were then suspended in individual organ chambers filled with Krebs buffer (5 mL) with the following millimolar composition: 118.5 NaCl, 25 NaHCO$_3$, 4.7 KCl, 1.2 MgSO$_4$·7H$_2$O, 1.2 KH$_2$PO$_4$, 11 glucose, and 2.5 CaCl$_2$·H$_2$O. The solution was aerated continuously with 95% O$_2$/5% CO$_2$ and maintained at 37°C. Isometric force was recorded with a tension transducer and data continuously collected (Biopac System). Aortic rings have been submitted to an optimal tension of 0.7 g which was readjusted every 15 min during a 60-min equilibration period. The vessels were left at this resting tension throughout the remainder of the study. Rings were repeatedly contracted with 60 mmol/L KCl until reproducible responses were obtained. Thereafter, vessels were incubated first for 30 min in presence or absence of Angl-7 (1 µM) with or without antagonists and further cumulative phenylephrine (PE) or AngII concentration-response curves have been constructed with cumulative incremental concentrations (1 nM to 1 mM).
Supplemental References


### Supplemental Tables

**Table S1.** Potency of RAAS peptides on AT1-R-mediated Gαq, Gαi3 and β-arr2 activation measured by BRET assays in HEK293T cells. Results were obtained from experimental data from Fig. 1B and 2B and represent the mean of EC$_{50}$ (nM) or pEC$_{50}$ ± S.E.M, n.a. not applicable.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>EC$_{50}$ (nM)</th>
<th>pEC$_{50}$ ± S.E.M</th>
<th>EC$_{50}$ (nM)</th>
<th>pEC$_{50}$ ± S.E.M</th>
<th>EC$_{50}$ (nM)</th>
<th>pEC$_{50}$ +/- S.E.M</th>
</tr>
</thead>
<tbody>
<tr>
<td>AngII</td>
<td>5.07</td>
<td>-8.30 ± 0.15</td>
<td>3.47</td>
<td>-8.46 ± 0.06</td>
<td>1.75</td>
<td>-8.76 ± 0.09</td>
</tr>
<tr>
<td>AngIII</td>
<td>1.81</td>
<td>-8.74 ± 0.09</td>
<td>1.47</td>
<td>-8.83 ± 0.06</td>
<td>6.18</td>
<td>-8.21 ± 0.12</td>
</tr>
<tr>
<td>AngIV</td>
<td>87.9</td>
<td>-7.06 ± 0.09</td>
<td>415</td>
<td>-6.38 ± 0.04</td>
<td>1732</td>
<td>-5.76 ± 0.23</td>
</tr>
<tr>
<td>Ang1-7</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>320</td>
<td>-6.49 ± 0.06</td>
</tr>
</tbody>
</table>

**Table S2.** Amino acid sequences of Angiotensin II and AngII-derived metabolites.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin II</td>
<td>Asp-Arg-Val-Tyr-Ile-His-Pro-Phe</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angiotensin III</td>
<td>Arg-Val-Tyr-Ile-His-Pro-Phe</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Angiotensin IV</td>
<td>Val-Tyr-Ile-His-Pro-Phe</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angiotensin 1-7</td>
<td>Asp-Arg-Val-Tyr-Ile-His-Pro</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

**Table S3.** Maximal efficacies (Emax) of RAAS peptides on BRET assays sensing AT1-R-mediated β-arrestin2 recruitment by measuring the interaction between AT1-R-Venus and RLuc8-β-arr2 or β-arr2-RLuc8. Results were obtained from experimental data shown in Fig. 4A and B and are expressed as the percentage of the maximal β-arrestin2 recruitment promoted by AngII and represent the mean ± S.E.M.. F values were obtained from a fit comparison (null hypothesis = One-phase association; alternative hypothesis = Two phase association). n.d. non determined

<table>
<thead>
<tr>
<th>Peptide</th>
<th>RLuc8-β-arr2</th>
<th>β-arr2-RLuc8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin II</td>
<td>100.0 ±8.8</td>
<td>100.0 ±1.1</td>
</tr>
<tr>
<td>Angiotensin III</td>
<td>92.5 ±2.8</td>
<td>102.1 ±1.4</td>
</tr>
<tr>
<td>Angiotensin IV</td>
<td>79.6 ±1.2</td>
<td>92.3 ±1.0</td>
</tr>
<tr>
<td>Angiotensin 1-7</td>
<td>76.1 ±0.7</td>
<td>72.8 ±1.1</td>
</tr>
</tbody>
</table>
Figure S1. Pharmacological characterization of RAAS peptides activity on AT1-R-mediated G protein activation using BRET assay. HEK293T cells co-expressing the indicated Gα-RLuc8 isoforms, GFP10-Gγ2 and Gβ1 in the presence or absence of HA-AT1-R were stimulated or not (vehicle) with 10 µM AngII, AngIII, AngIV or Ang1-7. Results are expressed as the difference in the BRET signal measured in the presence or absence of ligand. Data represent the mean ± S.E.M. of 3 to 4 independent experiments. Statistical significance of each ligand stimulation between HA-AT1-R-expressing and -non-expressing cells was assessed using an unpaired Student’s t-test (* P< 0.05).
Figure S2. BRET kinetics measurement of Gαq or Gαi3 activity following AT1-R stimulation with AngII or Ang1-7. HEK293T cells co-expressing Gαq-RLuc8 or Gαi3-RLuc8 with GFP10-Gγ2 and untagged Gβ1 isoform in the presence of HA-AT1-R were stimulated or not (vehicle) with 10 μM ligand for 2 or 10 min. Results are expressed as the difference in the BRET signal measured in the presence or absence of ligand. Data represent the mean ± S.E.M. of 3 to 4 independent experiments. Statistical significance between 2 min ligand stimulation and 10 min ligand stimulation was assessed using a paired Student's t-test (* P< 0.05).
Figure S3. Calcium production measurement in AT1-R-expressing HEK293T cells following AngII, AngIII, AngIV and Ang1-7 stimulation. HEK293T cells stably expressing mitochondrial-Aequorin and transiently expressing HA-AT1-R were stimulated with increasing concentrations of AngII, AngIII, AngIV or Ang1-7. Results represent the luminescence peak obtained following ligand stimulation and are expressed as the percentage of the maximal luminescence obtained in the presence of AngII. Data represent the mean ± S.E.M. of at least 4 independent experiments.
**Figure S4**

**A**

AT1-R / Gαq

-10 -9 -8 -7 -6 -5

0
20
40
60
80
100
120

AngII Ki = 6.1 \times 10^{-9} M

Ang1-7 Ki = 6.9 \times 10^{-7} M

**B**

AT1-R / Gαi3

-10 -9 -8 -7 -6 -5

0
20
40
60
80
100
120

AngII Ki = 3.1 \times 10^{-9} M

Ang1-7 Ki = 6.7 \times 10^{-7} M

**Figure S4.** Influence of Gαq or Gαi3 on AngII or Ang1-7 binding to AT1-R. Membranes prepared from HEK293T cells co-expressing the HA-AT1-R with either Gαq-RLuc8 (A) or the Gαi3-RLuc8 (B) with GFP10-Gγ2 and Gβ1 were incubated with [125I]AngII in the presence of increasing concentrations of AngII or Ang1-7. Binding is expressed as the percentage of specifically bound [125I]-Ang II. Results are expressed as mean ± S.E.M. of 3 independent experiments, each performed in triplicate. Ki values were obtained from competition curves analysis with GraphPad Prism v5 software.
**Figure S5.** AT1-R expression levels do not influence Ang1-7 antagonism of the AngII stimulated AT1-R/Gq and Gi axis. **Gaq (A)** or **Gi3 (B)** activation was measured by BRET in HEK293T cells co-expressing Gaq-FLuc8 or Gi3-FLuc8 with Gα1, GFP10-G;2 and different HA-AT1-R expression levels, stimulated or not with increasing concentrations of AngII for 2 min in the presence or absence of 10 μM Ang1-7. **(Upper panels)** HA-AT1-R cell surface expression levels were quantified in each transfection condition by ELISA using an anti-HA antibody. **(Middle panels)** Examples of AngII-dose responses BRET curves obtained with 3 different HA-AT1-R expression levels (a/ low expression, b/ medium expression, c/ high expression). Results are expressed as the difference in the BRET signal measured in the presence or absence of ligand and represent the mean ± S.E.M. **(Lower panels)** EC50s calculated from AngII-dose response curves (upper panels) in presence or absence of 10 μM Ang1-7 were plotted as a function of relative HA-AT1-R expression level. The EC50 of AngII are identical in the absence or presence of Ang1-7 whatever the AT1-R expression levels since the slope of the regression line is not significantly non-zero. Hence, the parallelism between the two regression lines reveals that receptor expression level does not influence the potency of Ang1-7 to antagonize AngII. Of note, Ang1-7 antagonism is significantly higher for AngII/Gi3 than for AngII/Gq (Unpaired t-test with welch’s correction P= 0.0035).
Figure S6. Ang1-7 antagonizes AngII-dependent calcium production by AT1-R. HEK293T cells stably expressing mitochondrial-Aequorin and transiently expressing HA-AT1-R were stimulated with increasing concentrations of AngII in the presence or not of 10 µM Ang1-7. Results represent the luminescence peak obtained following ligand stimulation and are expressed as the percentage of the maximal luminescence obtained in the presence of AngII. Data represent the mean ± S.E.M. of 4 independent experiments.
**Figure S7.** AT1-R expression levels do not influence Ang1-7 agonism on βarr2 recruitment to AT1-R. βarr2 recruitment to AT1-R was measured by BRET in HEK293T cells co-expressing a fixed βarr2-RLuc expression level but different HA-AT1-R-Venus levels in the presence of increasing concentrations of AngII or Ang1-7 for 15 min at 37°C. **(Upper panels)** HA-AT1-R cell surface expression levels were quantified in each transfection condition by ELISA using an anti-HA antibody. **(Middle panels)** Examples of AngII- or Ang1-7-dose responses curves obtained with 3 different HA-AT1-R expression levels (a/low expression, b/medium expression, c/high expression). Results are expressed as the difference in the BRET signal measured in the presence or absence of ligand and represent the mean ± S.E.M.. **(Lower panels)** EC50 calculated from AngII- and Ang1-7-dose response curves were determined and plotted as a function of relative HA-AT1-R expression level (OD). EC50 of AngII and Ang1-7 are identical whatever the receptor level since the slope of the two regression lines are not significantly non-zero.
Figure S8. Ang1-7 specifically recruits β-arr2 to AT1-R. BRET signals were recorded in HEK293T cells co-expressing HA-AT1-R-Venus and β-arr2-RLuc and stimulated or not during 15 min with increasing concentrations of Angll (left panel) or Ang1-7 (right panel) agonists in the presence or absence of 10 µM candesartan (Cande). Results are expressed as the difference in the BRET signal measured in the presence or absence of agonist. Data represent the mean ± S.E.M. of 3 to 5 independent experiments.
Figure S9. RAAS peptides dictate different AT1-R/β-arr2 trafficking pathways. (A, B) β-arr2 recruitment to AT1-R was visualized by confocal microscopy of HEK293T cells co-expressing βarr2-mCherry and HA-AT1-R-Venus that have been stimulated or not for 2 min (left panels), 5 min (middle panels) or 60 min (right panels) with 10 µM AngII, AngIII, AngIV or Ang1-7 as indicated. (A) Left panels images are representative merges of AT1-R-Venus (green), βarr2-mCherry (red) and nuclei (blue). Quadrants indicate the position where a 3x electronic magnification (middle, right panels) was carried out to outline the AT1-R/b-arr2 co-localization spots. Scale bar:10 µm.

(B) upper panel: Kinetics of β-arr2-mCherry and HA-AT1-R-Venus co-localization promoted by AngII, AngIII, AngIV or Ang1-7. Data represents mean ± S.E.M; lower panel: Co-localization coefficients were quantified for 15 individual cells (each square represents an individual cell) per condition over 3 independent experiments. Statistical significance of co-localization in the absence or presence of ligand as well as between ligands was assessed using one-way ANOVA followed by Dunn’s test (*** P<0.001, * P<0.05, n.s, not statistically significant, # P<0.001). All peptides promoted rapid β-arr2 recruitment to AT1-R with similar AT1-R/β-arr2 co-localization after 2 min treatment in agreement with the BRET results). However, over time, a different intracellular routing emerged between AT1-R and b-arr2 depending on the peptide that was visible from 5 min stimulation. While ~80% of internalized AT1-R still co-localized with β-arr2 after 60 min AngII and AngIII stimulation, only 40% co-localization was measured with AngIV and Ang-1-7. The mobilization of β-arr is dependent on the over-expressed AT1-R since no Angll-dependent punctate structure staining of β-arr was detected in the absence of AT1-R expression (Figure S10). These results most likely argue for AngIV and Ang-1-7 both acting as agonists for β-arr recruitment to the AT1-R, but with different β-arr recruitment modalities than those of AngII and AngIII.
Figure S10. AngII and Ang1-7 do not recruit β-arrestin2 in the absence of the AT1-R in HEK293T cells. β-arrestin2 recruitment was visualized by confocal microscopy on HEK293T cells co-expressing βarr2-mCherry in the absence of AT1-R transient expression, stimulated or not for 60 min with 10 µM AngII or Ang1-7, as indicated. Left images are a representative staining of βarr2-mCherry (red) while the right panel is the corresponding merge of βarr2-mCherry (red) and nuclei-DAPI (blue). Scale bar: 10 µm.
Figure S11. AT2-R and Mas-R gene expression in abdominal aorta from WT and AT1-R KO mice. Comparative qRT-PCR analysis of mRNA expression levels of Mas-R or AT2-R in total RNA extracted from 2-month old WT or AT1-R KO mice abdominal aorta. Data represent the mean ± S.E.M. of at least 4 mice. Statistical significance was assessed using unpaired 2-tailed Student's t test (n.s. non significant).
Figure S12. Effect of Ang1-7 on AngII-induced contraction of abdominal aorta. Contraction of abdominal aorta was promoted by incubation of aorta rings from WT (C57Bl/6) with different concentrations of AngII during 30 min pretreated or not (control-vehicle) with 1 µM Ang1-7 alone or in combination with 1 µM A-779 Mas-R antagonist. Data represent the mean ± S.E.M. of contractile response obtained in at least 4 mice. Statistical significance was assessed using two-ways ANOVA followed by Bonferroni test (*P < 0.05 compared with Ang1-7).