The Renin-Angiotensin System
Going Beyond the Classical Paradigms

Santos, Robson A Souza; Oudit, Gavin Y; Verano-Braga, Thiago; Canta, Giovanni; Steckelings, Ulrike Muscha; Bader, Michael

Published in:
American Journal of Physiology: Heart and Circulatory Physiology

DOI:
10.1152/ajpheart.00723.2018

Publication date:
2019

Document version
Accepted manuscript

Citation for published version (APA):

Terms of use
This work is brought to you by the University of Southern Denmark through the SDU Research Portal. Unless otherwise specified it has been shared according to the terms for self-archiving. If no other license is stated, these terms apply:

• You may download this work for personal use only.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying this open access version

If you believe that this document breaches copyright please contact us providing details and we will investigate your claim. Please direct all enquiries to puresupport@bib.sdu.dk
The Renin-Angiotensin System: Going Beyond the Classical Paradigms

Robson Augusto Souza Santos¹; Gavin Y. Oudit²; Thiago Verano-Braga¹; Giovanni Canta¹; Ulrike Muscha Steckelings³; Michael Bader⁴-⁸

¹National Institute of Science and Technology in Nanobiopharmaceutics, Department of Physiology and Biophysics, Federal University of Minas Gerais, Belo Horizonte-MG, Brazil.
²Division of Cardiology, Department of Medicine, Mazankowski Alberta Heart Institute, University of Alberta, Edmonton, Canada,
³IMM - Department of Cardiovascular & Renal Research, University of Southern Denmark, 5000 Odense C, Denmark.
⁴Max-Delbrück-Center for Molecular Medicine, Robert-Rössle-Str.10, D-13125 Berlin. ⁵DZHK (German Center for Cardiovascular Research), Partner Site Berlin.
⁶Berlin Institute of Health (BIH). ⁷Charité - University Medicine, Berlin, Germany.
⁸Institute for Biology, University of Lübeck, Lübeck, Germany

Running title: RAS: beyond the classics

Keywords: angiotensin-(1-7), angiotensin-(1-9), alamandine, angiotensin converting enzyme 2, heart failure.

*Corresponding author: Robson Augusto Souza Santos, MD, Ph.D. National Institute of Science and Technology in Nanobiopharmaceutics, Department of Physiology and Biophysics, Federal University of Minas Gerais. Av. Antonio Carlos 6627, Belo Horizonte-MG, Brazil. Phone: +55 31 3409 2956. Email: robsonsant@gmail.com
Abstract

Thirty years ago a novel axis of the renin-angiotensin system (RAS) was unveiled by the discovery of angiotensin (Ang)-(1-7) generation in-vivo. Later angiotensin-converting enzyme 2 (ACE2) was shown to be the main mediator of this reaction and Mas was found to be the receptor for the heptapeptide. The functional analysis of this novel axis of the RAS which followed its discovery revealed numerous protective actions in particular for cardiovascular diseases. In parallel, similar protective actions were also described for one of the two receptors of Ang II, AT2R, in contrast to the other, AT1R, which mediates deleterious actions of this peptide e.g. in the setting of cardiovascular disease. Very recently, another branch of the RAS was discovered, based on Ang peptides in which the aminoterminal aspartate is replaced by alanine, the alatensins. Ala-Ang-(1-7) or alamandine was shown to interact with the Mas-related receptor (MrgD) and first functional data indicate that this peptide also exerts protective effects in the cardiovascular system. This review summarizes the presentations given at the International Union of Physiological Sciences Congress in Rio de Janeiro, 2017, during the symposium “The renin-angiotensin system: going beyond the classical paradigms”, in which the signaling and the physiological actions of Ang-(1-7), ACE2, AT2R and the alatensins were reported (with a focus on non-CNS tissues) and the therapeutic opportunities based on these findings were discussed.
Introduction

The renin-angiotensin system (RAS) is one of the most potent cardiovascular regulators and an important target for therapeutic drugs. From 120 years ago, when renin was discovered (148) until 30 years ago only one peptide was thought to be active in this system, angiotensin (Ang) II. It was shown to be produced by successive digestion of angiotensinogen by the enzymes renin and angiotensin converting enzyme (ACE) with Ang I as intermediate and to interact with a receptor, now called AT_1 receptor (AT_1R). Physiologically, AT_1 activation by Ang II elicits vasoconstriction, water intake, and Na^+ retention. Pathophysiologically, activation of ACE/Ang II/AT_1R signaling is associated with oxidative stress, hypertrophy, fibrosis, and inflammation. This view of the RAS began to change when in 1988, Santos et al. discovered the formation of Ang-(1-7) from Ang I by an ACE-independent pathway (125), and Schiavone et al. showed the vasopressin releasing activity of this heptapeptide in neural tissue explants (131). One year later the first in-vivo actions of Ang-(1-7) were described (16). At about the same time, the second receptor for Ang II (AT_2 receptor) was found (21, 159). In the following 30 years until now the physiological functions of these two alternative pathways of the RAS have been extensively studied, and it was shown that Ang-(1-7) via its receptor Mas (127) as well as Ang II via AT_2R counteract the classical RAS resulting in vasodilation, anti-inflammation, anti-fibrosis and anti-apoptosis conferring beneficial effects in the settings of cardiovascular diseases (19, 128, 144). A central role in regulating the relative activities of the RAS arms in a tissue is played by the local levels of the enzyme ACE2 which transforms Ang II into Ang-(1-7) (31, 149). It was only a logical consequence of these findings that compounds were developed to activate the two protective arms of the RAS, which are undergoing clinical evaluation in the moment.
However, the full complement of Ang peptides was still not completed, since Lautner et al. (74) and Jankowsky et al. (60) recently discovered a novel class, which we suggest to call “alatensins”, with an alanine at the aminoterminal position instead of the aspartate in the classical Ang peptides. While Ala\(^1\)-Ang II interacts with the same receptors as Ang II, AT\(_1\)R and AT\(_2\)R, Ala\(^1\)-Ang-(1-7) (alamandine) has its own receptor, Mas-related G-protein coupled receptor D (MrgD) (74).

This review will summarize novel findings on the physiological and pathophysiological actions of the new beneficial RAS components, which were presented at the symposium on “The Renin-Angiotensin System: Going Beyond the Classical Paradigms” at the International Union of Physiological Sciences (IUPS) 2017 Rhythms of Life Congress in Rio de Janeiro.

Ang-(1-7) as a critical mediator of the ACE2-related protective axis of the RAS

Angiotensin-converting enzyme 2 (ACE2) is a monocarboxypeptidase that cleaves away phenylalanine from Ang II converting it to Ang-(1-7) and leucine from Ang I converting it to Ang-(1-9) (Figure 1A) (104, 109). Together, ACE2, Ang-(1-9), and Ang-(1-7) act as the endogenous negative regulator of the RAS. Ang-(1-7) is a biologically active heptapeptide exerting cardioprotective effects in the settings of cardiovascular diseases by antagonizing maladaptive signaling attributed to Ang II (1, 4, 88, 97). Ang-(1-7) primarily acts via the endogenous receptor Mas (Mas) since the Mas antagonist, A779, blocks the majority of Ang-(1-7) effects (1, 124, 127, 145, 171). More importantly, Ang-(1-7) effects are not observed in Mas-deficient animals (53, 127, 128, 164). The MrgD receptor can also mediate some of Ang-(1-7) action (127, 147). The AT\(_2\)R has been linked to cardioprotective actions of Ang-(1-9) (36, 37, 94,
The cardioprotective effects of ACE2 are a combination of (i) the degradation of Ang I to Ang-(1-9) thereby limiting the availability of the substrate for ACE and (ii) the degradation of Ang II to the cardioprotective Ang-(1-7). Therefore, the loss of ACE2 activity results in the loss of protection from maladaptive signaling of the Ang II/AT₁R axis promoting progression of cardiovascular diseases, whereas increased ACE2 activity leads to activation of ACE2/Ang-(1-7) and ACE2/Ang-(1-9) axes mediating cardiovascular protection (Figure 1B).

**Biochemistry and Regulation of ACE2**

ACE2 is a type I transmembrane protein consisting of an extracellular N-terminal domain containing the catalytic site and an intracellular C-terminal tail (31, 149). ACE2 functions as a carboxymonopeptidase with Arg²⁷³ residue being critical for substrate binding and enzymatic activity (49). As carboxymonopeptidase, ACE2 degrades Ang II to generate the heptapeptide Ang-(1-7) and Ang I to generate the nonapeptide Ang-(1-9) by hydrolyzing the C-terminal amino acid (31, 121, 149). Ang-(1-9) can be further converted to Ang-(1-7) by ACE. In the same manner, ACE2 can also cleave and partially inactivate pyr-apelin-13 and apelin-17 peptides (155). In addition to being an enzyme, ACE2 is a transmembrane protein that also acts as an amino acid transporter and is targeted by severe acute respiratory syndrome (SARS) coronavirus (109). ACE2 is widely expressed in the cardiovascular system, kidneys, lungs, and brain, to protect these organs from excessive Ang II signaling (32, 42, 54, 71, 99, 161). In the heart, both ACE2 and Mas are expressed in cardiomyocytes, cardiofibroblasts, and the coronary vasculature (58, 103, 108, 123, 124, 126). ACE2 activity is regulated by ADAM-17 (also known as TNFα converting enzyme – TACE) and apelin. ADAM-17 acts as a negative regulator of ACE2 tissue activity by
proteolytically cleaving ACE2 resulting in and shedding of ACE2 into the interstitium leading to a decreased ACE2 activity in the tissue and elevated circulating ACE2 activity (103, 109). Deletion of this negative regulator (ADAM-17 or TACE) protect the heart and vasculature in conditions of myocardial infarction, hypertension, pressure overload, and aortic aneurysm (34, 35, 135, 136). Unlike ADAM-17, apelin regulates ACE2 expression. In mice, knocking out of apelin is associated with lower ACE2 expression levels and exacerbated Ang-II-induced cardiac hypertrophy and dysfunction (172). Treatment of apelin knockouts with pyr1-apelin-13 normalized ACE2 expression and counteracted Ang-II-induced cardiac hypertrophy and dysfunction (172). Interestingly, ACE2 can also degrade and inactivate apelin peptides creating a self-regulating negative feedback loop (155).

**Generation and Metabolism of Ang-(1-7)**

Recombinant human ACE2 (rhACE) produces both Ang-(1-7) and Ang-(1-9) (from Ang II and Ang I, respectively) while recombinant murine ACE2 generates predominantly Ang-(1-7) from Ang II (6, 169). Studies using rhACE2 and ACE2 purified from sheep tissues showed that Ang II is a preferred substrate for ACE2 (48, 107, 134, 149, 153, 162, 173). In rats, changes in ACE2 correlated with plasma Ang-(1-9) levels (93) while rhACE2 efficiently converted infused Ang II into Ang-(1-7) (80). In mice, rhACE2 counteracts detrimental Ang II signaling, and these therapeutic effects are mediated via Ang-(1-7) (102, 107, 173). Also, in human studies, rhACE2 clearly lowered plasma Ang II levels and increased plasma Ang-(1-7) levels (6, 109). In the heart, Ang-(1-7) is primarily generated by ACE2 (41, 173), whereas plasma Ang-(1-7) is primarily produced by neprilysin (NEP) from Ang I since inhibition of NEP reduced Ang-(1-7) levels and increased Ang II levels (111). The degradation of Ang-
(1-7) is governed predominantly by the N-terminal catalytic domain of ACE, which degrades Ang-(1-7) to Ang-(1-5) (28). Inhibition of ACE increases plasma levels of Ang-(1-7) in both rodents and humans (3, 6, 20).

Role of ACE2 and Ang-(1-7) in heart failure

Heart failure (HF) is driven by activation of multiple neurohumoral and signaling pathways resulting in pathological hypertrophy and maladaptive ventricular remodeling. Activation of the RAS and increased Ang II levels play a pivotal role in adverse myocardial remodeling and disease progression (6, 85, 87) contributing to systolic and diastolic dysfunction in patients with HF (13, 57). Use of ACE inhibitors does not necessarily result in lower Ang II formation since Ang II levels can remain elevated in optimally treated HF patients. In fact, about 50% of the patients using current ACEi therapies exhibit elevated levels of Ang II, which is probably the result of increased Ang I levels and activity of mast cell chymase (6, 62, 78, 113, 158). This ACE-independent Ang II production highlights a need for a better understanding of the contribution of various parts of the RAS to HF progression.

Our current understanding of the role of various parts of the RAS in the development of HF is summarized in Figure 1B. In models of heart failure with reduced ejection fraction (HFrEF), loss of ACE2 worsened the heart failure phenotype (67, 102). Under the conditions of elevated Ang II levels, loss of protective ACE2 activity worsens cardiac dysfunction, hypertrophy, and fibrosis leading to greater diastolic dysfunction (2, 173). Supplying rhACE2 decreased plasma and myocardial Ang II levels and increased plasma Ang-(1-7) levels resulting in attenuated pathological remodeling and corrected diastolic dysfunction (173). Even partial loss of ACE2 (in heterozygote female mice) is sufficient to enhance the susceptibility to heart disease,
which is a clinically-relevant observation since ACE2 is downregulated in human hearts with dilated cardiomyopathy (156). Increase in soluble ACE2, which reflects loss of tissue-bound ACE2, is associated with severity of HF and serves as an independent predictor of major adverse cardiac events (6, 33). Since shedding of ACE2 is mediated via ADAM17 (59, 103, 163), it suggests a possible involvement of ADAM17 in development of HF. Involvement of ADAM17 in HF development has been shown primarily in relation to TNFα signaling pathway in humans (129, 130) and in mouse models of heart disease (34, 66). However, in the light of the findings that Ang II induces ADAM17-dependent cleavage of ACE2, it becomes clear that Ang II, ADAM17, and ACE2 create a potentially dangerous positive feedback loop conducive to development of HF (103) Conversely, loss of Ang-(1-7) action exacerbated heart disease in an ACE2-deficient state (102) while inhibition of Ang-(1-7)/Mas signaling prevented rhACE2-mediated cardioprotection (107) confirming the importance of Ang-(1-7) contribution to the cardioprotective effects of ACE2. Supplying Ang-(1-7) directly was also cardioprotective in preclinical models of heart disease (81, 88, 118, 145). Ang-(1-7) suppressed cardiomyocyte growth and myocardial-infarction-induced ventricular hypertrophy and decreased myocardial levels of pro-inflammatory cytokines leading to a reduction in myocardial inflammation (118, 145). Ang-(1-7) activation of Mas results in activation of phosphatidylinositol 3-kinase (PI3K)-Akt-endothelial nitric oxide synthase (eNOS), upregulation of mitogen-activated protein kinases (MAPK) phosphatase (84), inhibition of protein kinase C (PKC)-p38 MAPK and reactive oxygen species production (50, 102, 173), and suppression of collagen expression (reducing myocardial fibrosis) (29, 44, 124) thereby antagonizing pathological effects of Ang II (88, 109, 127). In addition to Ang-(1-7), Ang-(1-9), the product of ACE2 degradation of
Ang I, is anti-hypertrophic, anti-fibrotic, and anti-hypertensive in models of hypertension and myocardial infarction (36, 37, 94, 95).

ACE2 and Ang-(1-7) also play important roles in vascular disease and hypertension (109). For example, rhACE2 pretreatment partially reversed Ang II-mediated hypertension due to decreased plasma Ang II and increased plasma Ang-(1-7) levels (80, 162) while the loss of ACE2 exacerbates Ang II-mediated hypertension (108). Cyclodextrin-encapsulated Ang-(1-7) and the Mas agonists (AVE0091 and CGEN856S) have demonstrated anti-hypertensive effects in pre-clinical models (146). These anti-hypertensive effects of ACE2/Ang-(1-7) suggest new possible therapeutic options against hypertensive heart disease.

Role of ACE2/Ang-(1-7) in obesity-associated cardiomyopathy

Diabetes and obesity are major causes of cardiovascular morbidity and mortality worldwide and result in microvascular and macrovascular complications including hypertension (174). Diabetic heart disease, linked to systolic and diastolic dysfunctions, and HF (39, 47, 70, 174) is associated with activation of the RAS (11, 12, 56, 90). Obesity, the major metabolic precursor to type 2 diabetes, is an independent risk factor for the development of HF with preserved ejection fraction (68, 69, 98, 166). Emerging preclinical and clinical data strongly support a key pathogenic role for ACE2 and Ang-(1-7) in the progression of cardiovascular diseases (109, 132, 142). In the model of type 2 diabetes (db/db), Ang-(1-7) rescued the diastolic dysfunction, and reduced cardiac hypertrophy, fibrosis, lipotoxicity, and adipose inflammation (89, 100). Conversely, in the settings of high fat diet-induced obesity, loss of ACE2 worsened heart disease due to increased epicardial adipose tissue
inflammation, myocardial lipotoxicity, and worsened cardiac insulin resistance (105).

Ang-(1-7) prevented these changes and rescued HF with preserved ejection fraction (HFpEF) in ACE2 knockout mice (105). These findings coupled with the protective effects of ACE2 and Ang-(1-7) in the vasculature and adipose tissue, supports inflammation and microvascular dysfunction as key mediators of HFpEF (110, 116). These results suggest that, in HF patients with HFpEF, epicardial adipose tissue inflammation may be related to the cardiac dysfunction and adverse remodeling (105, 106). In obese patients, administration of Ang-(1-7) improved insulin-stimulated endothelium dependent vasodilation and blunted endothelin-1-dependent vasoconstrictor tone (132). Therefore, enhancing the ACE2 and Ang-(1-7) pathways represents a potential therapy for HFpEF, a condition with an adverse prognosis that lacks effective therapy.

Alatensins

In 2008 Jankowski and co-workers described a novel octapeptide, derived from Ang II, which they called Ang A. This peptide can be formed from Ang II by decarboxylation of the Asp¹ residue to Ala¹. The affinity of Ala¹-Ang II for AT₁ and AT₂ receptors, as determined by displacement of ¹²⁵I-Sar¹-Ile⁸-Ang II, was similar to Ang II. However, the pressor effect induced by Ang A was smaller than Ang II, suggesting the involvement of other mechanisms on this effect (60). This observation led to the hypothesis that reduced pressor effect was due to the formation of an Ang-(1-7)-like peptide (Ala¹-Ang-(1-7)) from Ang A. In pursuing this hypothesis, Santos and co-workers identified and characterized the heptapeptide Ala¹-Ang-(1-7), which they called alamandine (74). Differing from Ang A, which stimulates the same receptors as its precursor, alamandine did not act through Mas. Actually, alamandine
stimulates the Mas-related G-protein coupled receptor, MrgD (74). This receptor was initially believed to be an IB4-nociceptive neuron specific receptor (30, 76). However, there is growing evidence that MrgD is present in other tissues/cells including cardiomyocytes and endothelial cells (51, 61, 96). It is not clear yet if the lower pressor effect of Ang A as compared to Ang II is due to the formation of alamandine.

Although only two Ala\(^1\)-angiotensin related peptides have been reported, so far (i.e., Ang A and alamandine), we believe that this peptide family is larger than anticipated – probably containing Ala\(^1\)-Ang I, Ala\(^1\)-Ang-(1-9), Ala\(^1\)-Ang-(1-5), etc). To avoid confusion related to the angiotensin peptides, we are introducing the term “alatensins” to refer to the Ala\(^1\)-Angiotensin family. We are currently studying the physiological presence of these peptides using a LC-MS/MS platform with promising results that shall be published elsewhere soon.

**Physiological effects of alamandine**

Alamandine has been reported to reverse hyperhomocysteinemia–induced vascular dysfunction. Activation of PKA appears to be involved in this effect (117). In the adipose tissue an alamandine-induced reduction of leptin has been described through a mechanism involving Src/p38 MAP kinase (150). As previously described for Ang-(1-7), an inclusion compound of alamandine/HP-\(\beta\)-cyclodextrin reduced blood pressure in non-anesthetized spontaneous hypertensive rat (SHR). Likewise, as previously observed for Ang-(1-7) (83) oral administration of alamandine/ HP-\(\beta\)-cyclodextrin attenuated the cardiac pro-fibrotic effect of isoproterenol in Sprague Dawley (SD) rats (50). More recently, a cardioprotective effect of alamandine was described in an experimental model of sepsis (79). In keeping with these effects, it has been reported that knockdown of MrgD in mice leads to marked dilated
cardiomyopathy (96). Centrally the action of alamandine resembles those described for Ang-(1-7) in the RVLM, CVLM, and hypothalamus (38, 74, 137, 140). These observations suggest that, as described for Ang II and Ang-(1-7), alamandine may act as a neuronal excitatory molecule in the brain. However, striking differences between these two peptides are becoming progressively evident (61, 82). For instance, in the insular cortex, alamandine but not Ang-(1-7) promotes excitatory cardiovascular effects, including increase in blood pressure and heart rate associated to increased renal sympathetic activity (82). Moreover, Ang-(1-7) – and Ang II/AT$_2$R (112, 168) – promotes NO release mainly by activating the PI3K/AKT/eNOS pathway (124), while alamandine leads to NO release by a mechanism not involving this pathway (Figure 2). The AMPKa appears to be the main target for alamandine-induced NO formation (61).

It was well demonstrated by Jankowski and colleagues that Ang A binds and produces biological effects through interaction with AT$_1$R (60). This finding was confirmed by other authors (23, 167). However, the relationship of Ang A and AT$_1$R apparently is not straightforward as it appears. The vasoconstrictor effect of Ang A is smaller than Ang II, despite the fact that the affinity of both peptides for AT$_1$R is remarkably similar (60). This could be due to the formation of alamandine as we have hypothesized before (74). However, in isolated cardiomyocytes Ang A was essentially ineffective in promoting electrically stimulated Ca$^{++}$ influx (23). In the same preparation, Ang II was active. Accordingly, a 10-fold difference between Ang II and Ang A for intracellular calcium release in vascular smooth muscle cells was observed by Jankowski et al (60). These observations suggest that Ang A could be a biased agonist of AT$_1$R. This possibility has not been explored yet.
**Novel findings on Alamandine and AT_1R interaction**

Canta *et al.* (17) compared the effects of alamandine and Ang-(1-7) in non-anesthetized normotensive SD rats. Contrasting with Ang-(1-7), which has no effect on blood pressure, alamandine produced a dose-response U-shaped decrease in BP. The maximal decrease in BP was achieved by i.v. administration of 20 pg/animal. In order to test whether the U-shaped dose-response was due to stimulation of AT_1 receptors, the experiments were repeated in losartan-treated rats. In this condition, a magnification of the hypotensive response was observed (Figure 3). However, the U-shaped form was still evident. These results suggest that alamandine is more potent than Ang-(1-7) as a vasodilator and that AT_1R is involved on alamandine effects. A similar conclusion was drawn by Soltani Hekmat *et al.* in pentobarbital anesthetized rats (141). However, a more general conclusion about the modulatory role of AT_1R on the cardiovascular effects of alamandine cannot be drawn because in other rat strains (Wistar, SHR-SP) losartan abolished rather than increased alamandine vasorelaxant action (75). In addition, in the CVLM, blockade of AT_1R did not alter the hypotensive effect of alamandine (140).

**Novel downstream players of Ang-(1-7)/Mas and alamandine/MrgD signaling networks identified by phosphoproteomics**

Cell signaling is a communication process governing cellular actions and is mainly driven by reversible phosphorylation of downstream effectors. In the past decade, phosphoproteomics has emerged as a powerful approach to study phosphorylation dynamics. Figure 4 presents a general phosphoproteomic workflow to study cell signaling dynamics. This technology has been used to study the
signaling of Ang-(1-7)/Mas in human endothelial cells (152), and alamandine/MrgD signaling in CHO-MrgD and cancer cells (138).

**Ang-(1-7)/Mas signaling**

Since Ang-(1-7) was identified as the endogenous ligand of Mas in 2003 (127), western blot-based studies have contributed to build a solid knowledge of Ang-(1-7)/Mas signaling. For example, by using this method it was reported that Ang-(1-7)-induced NO production is dependent on the activation of PI3K/AKT/eNOS pathway (124), and that Ang-(1-7) activates SHP-2 to counter-regulate Ang II/AT₁ signaling (123).

Phosphoproteomics has been used to build a comprehensive time-resolved signaling network of Ang-(1-7) in human endothelial cells (152). Ang-(1-7) stimulation led to differential regulation of 121 phosphosites from 79 proteins including dephosphorylation of Ser²⁵⁶ on the transcription factor FOXO1 leading to its activation. FOXO1 is an important regulator of tumor suppression and cell metabolism (46, 72). FOXO1 activation by Ang-(1-7) seems to be an important molecular event on the antitumoral effect attributed to this heptapeptide. One could not anticipate that Ang-(1-7) would induce FOXO1 activation prior this phosphoproteomic study because AKT1 - a crucial player of Ang-(1-7) signaling (124) - induces FOXO1 phosphorylation and inhibition (15). Thus, this is a fine example of how phosphoproteomics can help one building an unbiased and comprehensive signaling network. Nevertheless, it is clear that other upstream players yet to be identified are responsible for FOXO1 dephosphorylation upon Mas activation.
Ang-(1-7)/Mas and Alamandine/MrgD induce NO formation via different signaling pathways

NO formation is a shared outcome of Ang-(1-7) and alamandine treatment. This event is associated with many beneficial effects observed for these peptides (45, 74). However, as mentioned before in the Alatensins section, Ang-(1-7) and alamandine does not trigger a common pathway to activate NOS (Figure 2). While Ang-(1-7)/Mas induces NO production via PI3K/AKT/eNOS pathway on endothelial cells and cardiomyocytes (29, 124), alamandine increases NO level in a LKB1/AMPK-dependent manner in cardiomyocytes. Using ventricular cardiomyocytes isolated from hearts of 10- to 12-wk-old male C57BL/6 mice, de Jesus and collaborators (61) showed that alamandine induced phosphorylation of AMPKα (Thr^{172}) and its upstream effector LKB1 (Ser^{428}). While Ang-(1-7) induced phosphorylation of AKT at its activation site Ser^{473}, the authors did not observe its phosphorylation using alamandine. Using cardiomyocytes from Mas-deficient mice, the authors ruled out the contribution of Mas in LKB1 and AMPKα phosphorylation induced by alamandine (61).

Although alamandine and Ang-(1-7) have 86% sequence identify (6 out of 7 amino acid residues are the same), the replacement of the negatively charged residue (Asp) to the neutral one (Ala) in position 1 changes the physicochemical feature of alamandine, influencing its receptor affinity as alamandine activates MrgD but not Mas (74). As reviewed in this manuscript, by activating different receptors, alamandine and Ang-(1-7) induce the activation of different signaling pathways, adding new therapeutic clues and insights for the understanding of the RAS.
The Angiotensin AT2-receptor: From enigma to therapeutics

In the late 1980ies, the development of new angiotensin receptor ligands by some pharmaceutical companies resulted in the realization that some of these ligands such as the Dupont compound DUP753 (the later losartan) or the Ciba-Geigy compound CGP42112A were able to distinguish between two different angiotensin receptor subtypes due to different affinity (21, 159). It soon became consensus to name the receptor, to which DUP753 bound with high affinity and CGP42112A with low affinity, angiotensin II type 1 receptor (now: AT1-receptor, AT1R) and the receptor, to which DUP753 bound with low affinity and CGP42112A with high affinity angiotensin II type 2 receptor (now: AT2-receptor, AT2R) (25).

Despite these new experimental tools for distinguishing between the AT1R and the AT2R, it took more than 5 more years until the scientific community began to really understand that AT1R and AT2R generally mediate opposing actions. There were several reasons, why this process took so long. For example, (i) CGP42112A was initially regarded to be an antagonist, which led to wrong interpretation of experimental data (159), (ii) for some time, several researchers regarded the AT2R as binding site without function (139) and (iii) as discussed in detail later in this article, G-protein coupling of the AT2R is quite unusual (170), which makes studies on its signaling and function difficult.

Signaling of the AT2-receptor and cross-talk with Mas

The mid 1990ies brought a real breakthrough in the understanding of the AT2R. Four groups independently discovered that activation of phosphatases seemed to be a major signaling mechanism of the AT2R, whereas the AT1R signals mainly through
kinase-driven signaling cascades. While Serge Bottari’s group found in 1992 that in general AT$_2$R activation leads to tyrosine dephosphorylation (9), in subsequent years, the groups of Colin Sumners, Victor Dzau and Clara Nahmias identified protein phosphatase 2 (PP2A) (63), MAP kinase phosphatase 1 (MKP-1) (165) and Src homology region 2 domain-containing phosphatase-1 (SHP-1) (92) as specific AT$_2$R-stimulated phosphatases. At the same time, it was excluded that the AT$_2$R couples to “conventional” G-proteins such as Gq- or Gs-proteins (10). However, coupling to Gi-proteins was demonstrated and was shown to be involved in the modulation of ion channel currents and activation of PP2A (55, 63). The lack of “conventional” G-protein coupling of the AT$_2$R was elegantly explained by a Nature publication from 2017, which reported the crystalline structure of the AT$_2$R (170). While this study confirmed that the AT$_2$R displays all characteristics of a 7-transmembrane, class A GPCR, the authors unexpectedly discovered that upon activation of the AT$_2$R, the intracellular helix 8 changes its orientation in a way that it interacts with the intracellular helices III, V and VI, thereby sterically blocking binding of conventional G-proteins and β-arrestins.

While these newest findings explain the lack of “conventional” G-protein coupling of the AT$_2$R, the actual signaling mechanisms of the receptor are still only incompletely understood. This holds in particular true for the initiation of signaling upon receptor activation. What is known, however, is that the third intracellular loop and the C-terminal end of the AT$_2$R seem crucial for AT$_2$R signaling (114, 115). In fact, some initial signaling molecules such as SHP-1, PP2A and the AT$_2$R interacting protein (ATIP) interact directly with the AT$_2$R upon receptor activation; SHP-1 and PP2A (probably under involvement of Gi) with the 3rd intracellular loop (64, 133) and ATIP
with the C-terminal end (8). Certain kinases may be involved, too, in this early initiation
of signaling such as the tyrosine kinase c-Src (133).

From a functional perspective, the lack of “conventional” G-protein signaling
and the activation of Gi and phosphatases, which again interfere with kinase driven
signaling in an inhibitory way, make sense and are in accordance with the known
AT₂R actions, which oppose actions of cytokines, growth factors and of classical
GPCRs such as the AT₁R (22, 24, 25, 122, 151, 157).

Signaling mechanisms of the AT₂R and Mas have many similarities like e.g.
involvement of SHP-1/SHP-2 and signaling through PI3K/AKT/eNOS (Figure 2).
Moreover, the AT₂R and Mas form heterodimers – at least in certain tissues – which
may explain the shared signaling pathways and also the phenomenon that often
effects of Ang-(1-7) can be inhibited by an AT₂R-antagonist, and effects of an AT₂R-
agonist by A779 (77, 101, 154). Interestingly, when the AT₂R and Mas dimerize, they
seem to functionally depend on each other, since knockout of one of the receptors
leads to loss of function of the other receptor in the respective cell or tissue (77).

Physiological and pathophysiological actions of the AT₂-receptor

In the physiological situation, the AT₂R is usually expressed at low levels and in
most tissues appears to be dormant (25). Exceptions seem to be a role in the central
regulation of blood pressure (143), a weak vasodilation (160), a natriuretic effect (52)
and an impact on cell differentiation e.g. in neurons (86), uterus (26) or fetal tissue
(18).

In the pathophysiological situation, the AT₂R mediates a variety of tissue
protective actions, which again resemble very much actions of Ang-(1-7) through Mas
and which comprise for example anti-inflammation, immune-modulation, anti-fibrosis,
inhibition of sympathetic outflow, anti-apoptosis and neuroregeneration (91). In the context of heart failure, several of these actions work together in a well-orchestrated way. For example, in rats with heart failure caused by myocardial infarction, $\text{AT}_2\text{R}$-stimulation acts anti-inflammatory by reducing cytokines synthesis and anti-fibrotic by inhibition of TGF-β generation, thus ameliorating peri-infarct remodeling, which again results in improved cardiac function (65, 73). An anti-fibrotic effect was also seen in right ventricles of rats with pulmonary hypertension (PH) (14). Right ventricular fibrosis leading to heart failure is in fact a major pathomechanism determining mortality in patients with PH. In another model of ischemia-induced heart failure (coronary ligation model), central administration of the $\text{AT}_2\text{R}$-agonist C21 for 7 days by i.c.v. infusion significantly reduced sympathetic outflow and improved baroreceptor sensitivity, both mechanisms with a proven beneficial effect on heart failure (40).

The protective effects of $\text{AT}_2\text{R}$-stimulation have been observed and associated with improved outcome in multiple other disease models, too, including cardiovascular disease, diabetic end organ damage, autoimmune disease, neurological disease etc. For more details on protective actions of the $\text{AT}_2\text{R}$ in a broad spectrum of diseases, the reader is referred to recent review articles (22, 24, 25, 27, 122, 151, 157).

**Targeting the $\text{AT}_2\text{R}$-receptor in drug development**

As a result of the better understanding of $\text{AT}_2\text{R}$ actions and the realization that the receptor promotes tissue protection, repair and regeneration in the context of several different pathologies, drug development projects have been initiated for the development of $\text{AT}_2\text{R}$ agonists. In the meantime, some of these projects have reached the clinical phases of development. The non-peptide $\text{AT}_2\text{R}$ agonist Compound 21
(C21), proprietary molecule of Vicore Pharma (Sweden; www.vicorepharma.com), has successfully undergone Phase I clinical testing and will soon be forwarded into a Phase IIa clinical study in patients with idiopathic pulmonary fibrosis. The cyclic peptide MOR107 (Morphosys, Germany; www.morphosys.com; previously LP-2 by Lanthio Pharma, Netherlands; www.lanthiopharma.com) is currently tested in a Phase I clinical trial, while the clinical testing of MP-157 (Mitsubishi Tanabe, Japan; www.mt-pharma.co.jp) has been discontinued. The AT\(_2\)R antagonist EMA401 (Novartis, Switzerland; www.novartisclinicaltrials.com; previously Spinifex, Australia) has been successfully tested in a Phase II trial for the treatment of neuropathic pain (120). More Phase II studies with EMA401 are currently initiated.

**Conclusions and Future Directions**

The numerous beneficial actions of the novel RAS arms summarized in this review warrant clinical verification and therapeutic exploitation.

ACE2 has emerged as the dominant mechanism for negative regulation of the RAS, by metabolizing Ang II into the beneficial peptide Ang-(1-7). This heptapeptide has emerged as major protective peptide in the cardiovascular system. Clinical and experimental findings demonstrated that ACE2 and Ang-(1-7) comprise the dominant mechanism for protective regulation of the RAS in many types of HF. Ang-(1-7) generated by ACE2-dependent conversion of Ang II is a crucial mediator of the cardioprotective effects, which makes Ang-(1-7) a promising therapy for HF.

After a long period of preclinical research in order to better understand AT\(_2\)R signaling and function, this knowledge is currently being translated into developments for a potential, future clinical use of drugs targeting the AT\(_2\)R. Most of these drugs in development are AT\(_2\)R agonists with current primary indications being fibrotic diseases
and diabetic nephropathy. The AT$_2$R antagonist EMA401 is developed for the treatment of neuropathic pain. Phase II clinical studies are currently being initiated and will provide information about the therapeutic potential of drugs targeting the AT$_2$R.

First compounds activating the Mas and AT$_2$ axis are available and clinical trials have been started. For alatensins the physiological and pathophysiological importance has still to be confirmed before therapeutic approaches can be initiated. However, it is quite likely that we will soon have novel therapies for cardiovascular and other diseases based on the beneficial RAS peptides. Indeed, a recent study described beneficial effects of the oral formulation hydroxypropyl-β-cyclodextrin/Ang-(1-7) in volunteers submitted to isometric overload muscle damage (7). This study which is the first to test the effects of an oral formulation of Ang-(1-7) in humans opens new possibilities for testing Ang-(1-7) actions and therapeutic effects in patients.

Finally, this review highlights the potential of phosphoproteomics to investigate Ang-(1-7) and alamandine signaling in an unbiased way, allowing the identification of unanticipated downstream effectors of these signaling networks.

**Sources of Funding**

We acknowledge funding support from the Canadian Institutes of Health Research and Heart & Stroke Foundation to GYO, and the Brazilian Funding Agencies: CNPq (TVB, 421021/2016-0), FAPEMIG (RAS, APQ-03139-16 and TVB, APQ-03242-16) and CAPES to RAS and TVB.

**Disclosures**

NONE.
References:


**Figure Legend**

**Figure 1.** Enzymatic cascade involving the renin-angiotensin system, key receptor systems, and the biological effects. (A) Renin-angiotensin system cascade showing the angiotensin peptide metabolic pathway. Ang I is cleaved by ACE to Ang II, which is metabolized by ACE2 to Ang-(1-7). Ang II binds to AT$_1$ and AT$_2$ receptors, whereas Ang-(1-7) binds to Mas receptors and oppose Ang II/AT$_1$R actions. (B) Decreased ACE2 shifts the balance in the renin-angiotensin system towards the Ang II/AT$_1$R axis resulting in cardiovascular disease progression. Increased ACE2 shifts the balance to Ang-(1-7)/Mas axis leading to protection from cardiovascular disease.

ACE: angiotensin-converting enzyme; ACE2: angiotensin-converting enzyme 2; ADH: antidiuretic hormone; Ang: angiotensin; APA: aminopeptidase A; AT$_1$R: Ang II type 1 receptor; AT$_2$R: Ang II type 2 receptor; MasR: Ang-(1-7) receptor; PCP: prolyl carboxypeptidase (also known as angiotensinase C). Reproduced with permission from (109).

**Figure 2.** Signaling pathways for NO formation. Ang-(1-7)/Mas and Ang II/AT$_2$R induce NO formation via PI3K/AKT signaling while alamandine/MrgD leads to NO formation through AMPK activation.

**Figure 3.** Effect of alamandine on blood pressure in non-anesthetized 12 weeks old Sprague Dawley male rats. Administration of drugs and the blood pressure measurement were performed with a cannula inserted in the femoral vein and artery, respectively. Alamandine was administered i.v. *in bolus* in increasing amounts (0.02, 0.2, 1, 5, 20 and 80 ng). Subsequent alamandine amount was administrated only when MAP returned to basal level. Losartan (5mg/Kg) was administrated *i.v. in bolus* 30 minutes before alamandine i.v. administration. A U-shaped effect of alamandine was observed. *$p < 0.05$ vs saline. #$p < 0.05$ vs alamandine. Statistical significance
was obtained by two-way analysis of variance (ANOVA) followed by Bonferroni. Each bar represents the mean ± SEM. MAP: mean arterial pressure.

**Figure 4.** Phosphoproteomic approach to study signaling pathways. A) Activation or inhibition of a given receptor leads to phosphorylation and dephosphorylation of target proteins from plasma membrane towards nucleus in a time-dependent manner. B) General phosphoproteomic workflow for temporal cell signaling studies. (i) To study phosphorylation and dephosphorylation dynamics, experimental groups are divided based on treatment duration (e.g., from seconds to days). Untreated cells are used as control and are considered time = 0 minutes. (ii) Cells are lysed to extract proteins and phosphoproteins and then digested using specific proteases (e.g., trypsin). (iii) Generated peptides are labelled with non-radioactive isotopes for multiplexing analysis. (iv) Phosphopeptides are enriched to reduce sample dynamic range. (v) Samples are analyzed using liquid chromatography coupled to mass spectrometry (LC-MS) and bioinformatics tools are used to identify and quantify phosphoproteins, including their phosphorylation sites, to ultimately build dynamic signaling networks affected by the given treatment.
Alamandine

Saline

Alamandine + Losartan (5mg/kg)

\[ \Delta \text{MAP (mmHg)} \]

-25 -20 -15 -10 -5 0 5 10 15

-25 -20 -15 -10 -5 0 5 10 15

ng

Saline

Alamandine

Alamandine + Losartan (5mg/kg)
(A) Ligand binding to cell membrane proteins, generating phosphorylation sites.

(B) Flowchart:

i. Control and Treatment conditions over time.

ii. Cell lysis and protein digestion.

iii. Peptide labeling.

iv. Phosphopeptide enrichment.

v. LC-MS analysis and bioinformatics.