Counter-regulatory renin–angiotensin system in cardiovascular disease

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Abstract | The renin–angiotensin system is an important component of the cardiovascular system. Mounting evidence suggests that the metabolic products of angiotensin I and II — initially thought to be biologically inactive — have key roles in cardiovascular physiology and pathophysiology. This non-canonical axis of the renin–angiotensin system consists of angiotensin 1–7, angiotensin 1–9, angiotensin-converting enzyme 2, the type 2 angiotensin II receptor (AT_2R), the proto-oncogene Mas receptor and the Mas-related G protein-coupled receptor member D. Each of these components has been shown to counteract the effects of the classical renin–angiotensin system. This counter-regulatory renin–angiotensin system has a central role in the pathogenesis and development of various cardiovascular diseases and, therefore, represents a potential therapeutic target. In this Review, we provide the latest insights into the complexity and interplay of the components of the non-canonical renin–angiotensin system, and discuss the function and therapeutic potential of targeting this system to treat cardiovascular disease.

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The renin–angiotensin system (RAS) has a critical role in cardiovascular physiology through its effects in regulating blood pressure and electrolyte balance[1]. However, under pathophysiological conditions, the effects of the RAS can intensify to trigger inflammation and structural remodelling, thus promoting cardiac and vascular damage[2,3]. Researchers have studied the RAS for more than a century, not only to understand its role in normal physiological function but also to develop effective therapies to treat its dysregulation[1,2]. These systematic research efforts have led to the discovery of a non-canonical RAS, which has challenged the hypothesis that the RAS can only exert deleterious effects on the cardiovascular and renal systems. In the classical system, renin cleaves angiotensinogen to form angiotensin I, which is subsequently converted to angiotensin II by angiotensin-converting enzyme (ACE) (FIG. 1). Conversely, ACE2 can cleave angiotensin II to produce angiotensin 1–7, and can cleave angiotensin I to generate angiotensin 1–9[1,3]. Increasing evidence supports the concept that these systems work to produce opposite effects, suggesting a counter-balancing role for the two axes in cardiovascular physiology and disease. A timeline of key historical findings associated with the study and discovery of the counter-regulatory RAS is shown in BOX 1. In light of the emergence of multiple studies evaluating the effects and signalling pathways elicited by the counter-regulatory RAS in the past decade, we sought to provide an update on the current understanding of the complex regulation of the non-canonical RAS. In this Review, we discuss the cardioprotective effects of the non-canonical RAS and provide a critical analysis of the current challenges that must be overcome to translate its therapeutic effects into the clinical context.

Components of the counter-regulatory RAS

Ligands

The counter-regulatory RAS is made up of various peptides, receptors and enzymes (FIG. 1). Whereas the effects of angiotensin 1–7 and angiotensin 1–9 on the cardiovascular system have been explored previously[3], the potential roles of other counter-regulatory RAS components remain poorly understood. These non-canonical RAS components include alamandine, angiotensin 1–12 and angiotensin 1–5, as well as angiotensin 2–8 and angiotensin 3–8, which are also known as angiotensin III and IV, respectively[3]. FIGURE 2 shows the molecular structures of these peptides.

In the past 10 years, new evidence has emerged about the signalling pathways triggered by the counter-regulatory RAS, revealing their role as potential therapeutic targets for cardiovascular disease (CVD). Angiotensin 1–7 can act as a β-arrestin-biased agonist of the type 1 angiotensin II receptor (AT_1R) without activating the G_q subunit. This mechanism might contribute to the anti-hypertrophic properties of angiotensin 1–7, given that neither activation of the AT_1R nor the proto-oncogene Mas receptor antagonists prevented the beneficial effects of this peptide[4]. Alamandine activates the AMP-activated protein kinase (AMPK)–nitric oxide (NO)

Key points

- Chronic activation of the renin–angiotensin system (RAS) promotes cardiovascular damage, an effect that is antagonized by components of the counter-regulatory RAS.
- Components of the counter-regulatory RAS, including angiotensin 1–7, angiotensin 1–9, alamandine and their receptors have been found to be protective in multiple cardiovascular diseases, such as hypertension and heart failure.
- Numerous preclinical studies have demonstrated the beneficial effects of the counter-regulatory RAS, but clinical trials confirming these observations are still scarce.
- The challenges in quantitating angiotensin 1–7, angiotensin 1–9 and alamandine associated with their short plasma half-life and similarity in their molecular structures must be overcome before these peptides can be evaluated in the clinical setting.

pathway via the Mas-related G protein-coupled receptor member D (MRGD), which prevents angiotensin II-induced hypertrophy[5]. By contrast, angiotensin 1–9 stimulates the AT_2R–AKT signalling pathway to protect the myocardium against reperfusion-induced cell death[6]. Moreover, angiotensin 1–12 has been shown to regulate intracellular calcium transients and left ventricular contractile function in both normal rats and rats with heart failure (HF) via a chymase-dependent and cyclic AMP-dependent mechanism[7]. Additionally, angiotensin 1–5 has been found to induce atrial natriuretic peptide (ANP) secretion from isolated perfused rat atria by binding to the Mas receptor and activating the phosphatidylinositol 3-kinase–AKT–endothelial NO synthase pathway[8]. Lastly, angiotensinogen is the precursor for the entire RAS family of peptides, but, to date, no studies have shown that angiotensinogen can elicit direct biological effects on the heart. Nonetheless, the aryl hydrocarbon receptor nuclear translocator-like protein 1 has been shown to modulate blood pressure through a mechanism involving transcriptional regulation of angiotensinogen in a circadian manner in perivascular adipose tissue, which in turn increases local angiotensin II production[9]. These novel findings shed light on the complex regulation of the classical RAS and suggest a similar complexity for its counter-regulatory system. In this context, circadian expression of local angiotensinogen might affect organ-specific activity of peptides with known cardiovascular effects, such as angiotensin 1–7 or angiotensin 1–9, and requires further investigation.

Receptors

In the non-canonical RAS, angiotensin 1–7 and angiotensin 1–9 bind to the Mas receptor and AT_2R, respectively, whereas alamandine acts through the MRGD[3] (FIG. 3). Angiotensin 1–7 can also bind to the MRGD, but the functional relevance of this association remains unclear[10]. Emerging evidence reveals a more complex interaction between components of the classical and the counter-regulatory RAS than initially thought, given that angiotensin 1–7 has also been shown to bind to AT_2R[11]. Moreover, AT_1R can form heterodimers with the Mas receptor, which inhibits the activity of AT_1R[12]. Using radiolabelling and dynamic mass redistribution experiments in cells overexpressing the Mas receptor, Gaidarov and colleagues found that although angiotensin 1–7 can antagonize angiotensin II signalling, angiotensin 1–7 does not bind directly to the Mas receptor[13]. These data conflict with an earlier study that demonstrated binding of fluorescent or ^{125}I-labelled angiotensin 1–7 to the Mas receptor[14]. Gaidarov and colleagues noted that in the absence of the Mas receptor, angiotensin 1–7 has no effect on angiotensin II signalling[13]. However, the investigators also reiterated that rigorously controlled experiments demonstrating interactions between angiotensin 1–7 and the Mas receptor are very scarce. Moreover, given that their findings suggest that angiotensin 1–7 does not bind to the Mas receptor, the researchers hypothesized that any cardioprotective effects of angiotensin 1–7 might be attributable to antagonism of angiotensin II signalling[13]. Nevertheless, additional studies are required to confirm whether angiotensin 1–7 is an endogenous agonist of the Mas receptor.

Meems and colleagues designed and synthesized NPA7, a peptide that can simultaneously activate the Mas receptor and the particulate guanylyl cyclase A receptor[15]. NPA7, generated by the fusion of angiotensin 1–7 with a 22-amino acid sequence of the B-type natriuretic peptide (BNP), reduced blood pressure, cardiac unloading and systemic vascular resistance, and exerted a more potent natriuretic and diuretic effect than separate administration of BNP and angiotensin 1–7. These observations raise the possibility that fusion of other counter-regulatory RAS ligands that can target more than one receptor might also induce a synergistic effect to mediate potent cardioprotective benefits. AT_2R can form functional heterodimers with Mas receptors, highlighting the possibility of developing drugs that can selectively target monomers or oligomers to upregulate or downregulate specific cell signalling cascades in the cardiovascular system[16]. In addition, the crystal structures of human AT_2R bound to a selective ligand indicate that the ligand can induce an active conformation of the receptor, suggesting that AT_2R does not bind to G proteins or β-arrestins[17]. Tetzner and colleagues showed that angiotensin 1–7 can bind to the MRGD and that the AT_2R antagonist PD123319 can block both the Mas receptor and MRGD[10]. This latter finding is particularly important, given the large number of studies utilizing PD123319 to assess the effects of AT_2R activation. FIGURE 3 provides an overview of the signalling pathways triggered by the counter-regulatory RAS ligands upon binding to their receptors.

Regulatory enzymes

ACE inhibitors are a first-line pharmacological therapy in the management of hypertension. Other proteases such as ACE2 and neprilysin (also known as neutral endopeptidase) have been identified as novel therapeutic targets, given that these enzymes can also reduce blood pressure. ACE2 might reduce blood pressure levels by generating angiotensin 1–7 from angiotensin II, whereas inhibition of neprilysin increases ANP levels[18]. In addition, the endogenous metabolic regulator fibroblast growth factor 21 (FGF21) can promote ACE2 generation in adipocytes and renal cells, thereby promoting the cleavage of angiotensin II to form angiotensin 1–7, suggesting that FGF21 can reduce angiotensin II-induced hypertension[19].

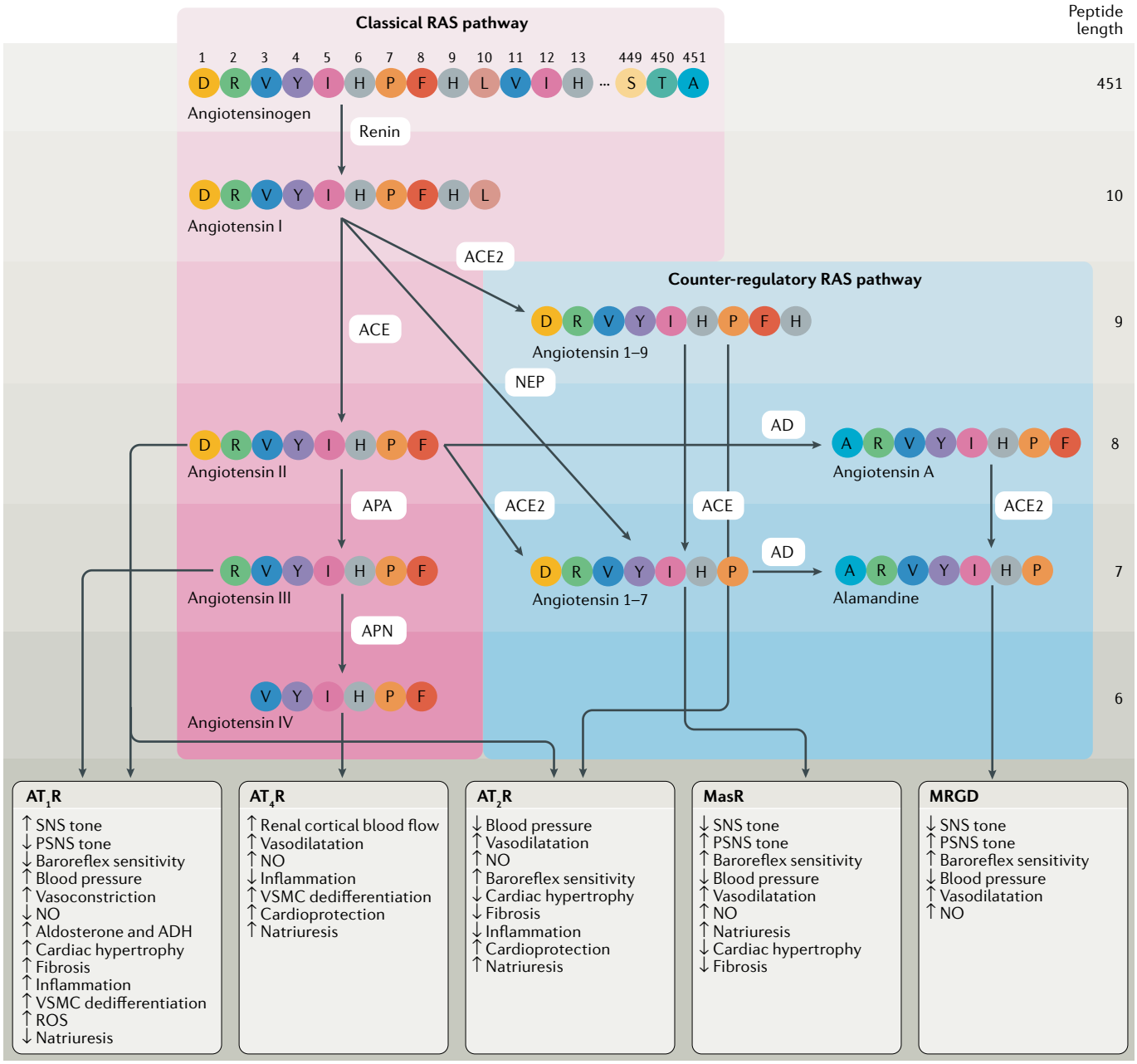
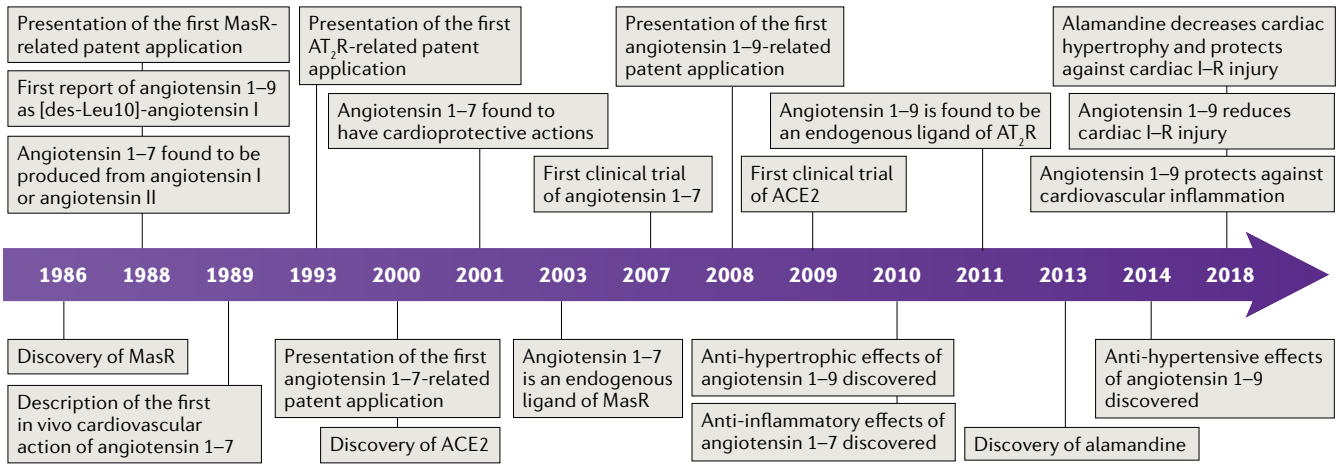


Fig. 1 | **Classical and counter-regulatory renin–angiotensin pathways.** In the classical system, renin cleaves angiotensinogen to produce angiotensin I. This peptide can be processed by angiotensin-converting enzyme (ACE) to form angiotensin II, which in turn can bind to the type 1 angiotensin II receptor (AT_1R) and AT_2R[3]. AT_1R activation increases aldosterone[165] and anti-diuretic hormone (ADH)[166] production, sympathetic nervous system (SNS) tone[167], blood pressure[168], vasoconstriction[169], cardiac hypertrophy[170], fibrosis[171], inflammation[172], vascular smooth muscle cell (VSMC) dedifferentiation[173] and reactive oxygen species (ROS) production[36], while decreasing parasympathetic nervous system (PSNS) tone[174], baroreflex sensitivity[175], nitric oxide (NO) production[176] and natriuresis[177]. Angiotensin II can be further processed by aminopeptidase A (APA) to form angiotensin III, which also acts through AT_1R. Angiotensin III can be cleaved by alanyl aminopeptidase N (APN) to generate angiotensin IV, which binds to AT_4R, producing cardioprotective effects[178], increasing natriuresis[179] and NO production[180], as well as reducing vasoconstriction[181], inflammation[178] and VSMC dedifferentiation[182]. Angiotensin I can also be cleaved by ACE2 and neprilysin (NEP) to produce angiotensin 1–9 and angiotensin 1–7, respectively[3]. Angiotensin 1–9 can activate AT_2R to trigger natriuresis[183] and NO production[73], thus mediating vasodilatory effects[73] and reducing blood pressure[73]. In addition, this peptide is cardioprotective[6] and can attenuate inflammation[73], cardiac hypertrophy[135] and fibrosis[73]. Angiotensin 1–7 binds to the proto-oncogene Mas receptor (MasR) and reduces both blood pressure[184] and noradrenaline release in hypertensive rodents[185]. Conversely, activation of MasR increases NO generation[186], natriuresis[187], vasodilatation[186], PSNS tone and baroreflex sensitivity[188,189]. Angiotensin 1–7 can also be formed from angiotensin II cleavage by ACE2 and be further metabolized to alamandine. Alternatively, angiotensin II can be processed by aspartate decarboxylase (AD) to produce angiotensin A, which can be converted to alamandine by ACE2. Upon binding to the Mas-related G protein-coupled receptor member D (MRGD), alamandine can promote the same effects reported for angiotensin 1–7[5,67,190], with the exception of natriuresis. RAS, renin–angiotensin system.

Box 1 | **Timeline of the discoveries related to the counter-regulatory RAS**

The timeline in the figure shows a historical perspective of the most important findings associated with the counter-regulatory renin–angiotensin system (RAS). The proto-oncogene Mas receptor (MasR) was initially described as an oncogene and detected through its tumorigenicity in nude mice[147]. Angiotensin 1–9 and angiotensin 1–7 were first identified from hydrolytic cleavage of angiotensin I, and angiotensin I or angiotensin II, respectively[148,149]. In 1989, angiotensin 1–7 was found to have anti-hypertensive effects in rats upon unilateral injection into the medial "nucleus of the solitary tract" and into the dorsal motor nucleus of the vagus[150]. The earliest patents related to the components of the counter-regulatory RAS described the use of the MasR in an assay system for detecting angiotensin-blocking activity, a cDNA encoding the type 2 angiotensin II receptor (AT_2R) in mice and rats, a nucleic acid encoding angiotensin 1–7, a cDNA encoding angiotensin-converting enzyme 2 (ACE2) and the use of angiotensin 1–9 to prevent, reverse, inhibit or reduce cardiovascular, pulmonary, cerebral or renal remodelling. ACE2 was simultaneously discovered by two independent research groups in 2000[151,152]. Angiotensin 1–7 was subsequently described as a cardioprotective peptide[153] with anti-inflammatory actions[110] and found to be activated through the MasR[14]. The first clinical trial of angiotensin 1–7 assessed the effect of this peptide on the reduction of blood flow in solid tumours[154], whereas the first trial of ACE2 evaluated the safety and tolerability of a recombinant form of ACE2[121]. The anti-hypertrophic[135], anti-hypertensive[73], anti-inflammatory[120] and cardioprotective[6] properties of angiotensin 1–9 have been described. Alamandine was discovered in 2013 as an anti-hypertensive agent[67], and the cardioprotective properties of this compound have since been described[155,156].



I–R, ischaemia–reperfusion.

Intercellular communication

The classical RAS can act at both local and systemic levels, but how these signals are coordinated is poorly understood. Exosomes, which are extracellular vesicles of 50–100 nm in size, can transport and transmit molecules such as proteins and microRNAs from one cell to another, and can also transport components of the classical RAS[20]. Previously assumed to be scattered cellular waste, exosomes are attracting much research interest since the discovery of their role in intercellular communication[21]. Considering that these extracellular vesicles can communicate signals from afar and that the counter-regulatory RAS can exert its effects on multiple cell types, these vesicles might have a role in orchestrating the effects of the counter-regulatory RAS. In this context, Pironti and colleagues observed that exosomes induced by cardiac pressure overload in mice contain functional AT_1R, which might influence AT_1R-mediated regulation of vascular tone[22]. Moreover, exosomes seem to have a role in the local RAS. Angiotensin II triggers exosome production in rat cardiac fibroblasts in vitro, and these exosomes in turn promote angiotensin II production and AT_1R expression in rat cardiomyocytes in vitro, suggesting a positive feedback mechanism that might contribute to the exacerbation of cardiac hypertrophy elicited by angiotensin II[23]. However, this evidence only supports a role for exosomes in orchestrating the effects of the canonical RAS. Whether extracellular vesicles contribute to the cardioprotective properties of the counter-regulatory RAS remains to be determined.

Counter-regulatory RAS in CVD

Pulmonary arterial hypertension

The ACE2–angiotensin 1–7–Mas receptor axis. ACE2, first described as a receptor for severe acute respiratory syndrome coronavirus, is characterized by its marked homology with ACE[24]. The therapeutic potential of ACE2 agonists for pulmonary arterial hypertension (PAH) has been explored in a number of studies. In rats with monocrotaline-induced PAH, *Ace2* gene therapy prevented PAH-mediated hypertrophy and functional impairment of the right ventricle[25]. Moreover, synthetic activators of ACE2 (XNT[26] and resorcinolnaphthalein[27]) improve pulmonary artery endothelial function by inducing phosphorylation of endothelial NO synthase at Ser1177 and dephosphorylation at Thr495[27], which consequently increases the bioavailability of NO. A meta-analysis to assess the efficacy of 522 interventions for PAH revealed that these ACE2 synthetic activators were among the most potent agents[28]. Although these findings strongly support the therapeutic potential of ACE2 activators, translation of these agents into a clinical setting remains challenging because ACE2 is a membrane-bound enzyme. ACE2 can be cleaved and its soluble and catalytically active form can be secreted[29,30]. Given that increasing the circulating levels

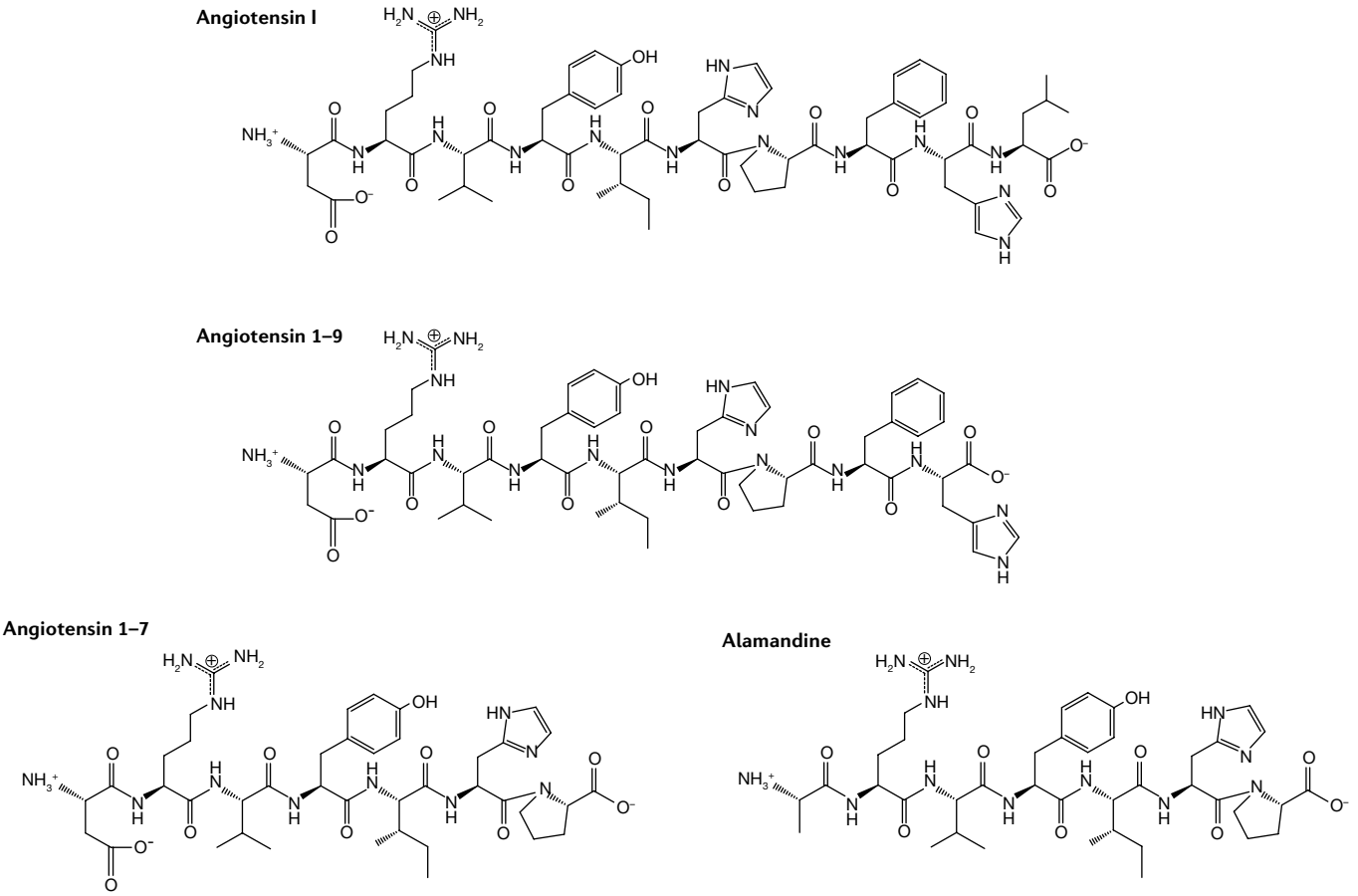


Fig. 2 | **Molecular structures of peptides of the counter-regulatory RAS.** The separation of these peptides from a biological sample is difficult, given the similarity of their molecular structures. Angiotensin 1–7 is only two amino acids shorter than angiotensin 1–9, and angiotensin 1–7 and alamandine only differ in their N-terminal amino acid. RAS, renin–angiotensin system.

of ACE2 might have a therapeutic effect, a recombinant human ACE2 (rhACE2) has been developed and tested in animal models. Administration of rhACE2 improved right ventricular function in mice subjected to pressure overload[31] and attenuated vascular remodelling in mice with bleomycin-induced pulmonary hypertension[32]. A pilot study evaluated the effects of increasing the enzymatic activity of ACE2 through intravenous infusion of 0.2 mg/kg or 0.4 mg/kg of rhACE2 in patients with PAH[33]. The drug was well tolerated and had beneficial effects on pulmonary vascular resistance and cardiac output, in addition to reducing inflammatory markers and increasing superoxide dismutase 2 levels in plasma. Nonetheless, this proof-of-concept study included only five patients. In a separate study, rhACE2 administration was also shown to be well tolerated in 44 patients with acute respiratory distress syndrome[34]. The safety profile of rhACE2 needs to be further assessed in clinical studies.

Angiotensin 1–7 and other Mas receptor activators might also have a protective role against the development of PAH[35]. Notably, however, angiotensin 1–7 is not considered a good therapeutic candidate owing to its pharmacokinetic limitations. Angiotensin 1–7 is rapidly cleaved by peptidases and thus has a very short half-life of ~10 s (REF.[36]). However, cell signalling mechanisms and effects mediated by biological peptides are thought to persist despite their short half-life[37]. Furthermore, studies in animal models have shown that administration of angiotensin 1–7 included in cyclodextrin complexes has neuroprotective effects and improves muscle damage induced by eccentric cardiac overload[38–44]. A stable, cyclic analogue of angiotensin 1–7 moderately reduced right ventricular systolic pressure in a rat model of monocrotaline-induced PAH, but no significant changes were observed in the medial wall thickness of pulmonary arterioles[45]. To optimize the protective potential of this angiotensin 1–7 analogue for the treatment of PAH, the compound can potentially be combined with a neprilysin inhibitor or an ACE2 activator[46]; whether this approach is effective in maintaining high levels of angiotensin 1–7 requires further investigation.

AT_2R stimulation. AT_2R activation can attenuate right ventricular and pulmonary remodelling[47]. AT_2R stimulation protected mice from severe lung injury induced by sepsis or acid aspiration[48], whereas AT_2R deficiency exacerbated HF in mice subjected to acute myocardial infarction[49]. Furthermore, activation of AT_2R (using the agonist dKc-angiotensin 1–7) in a rat model of chronic lung disease protected the heart and lungs from

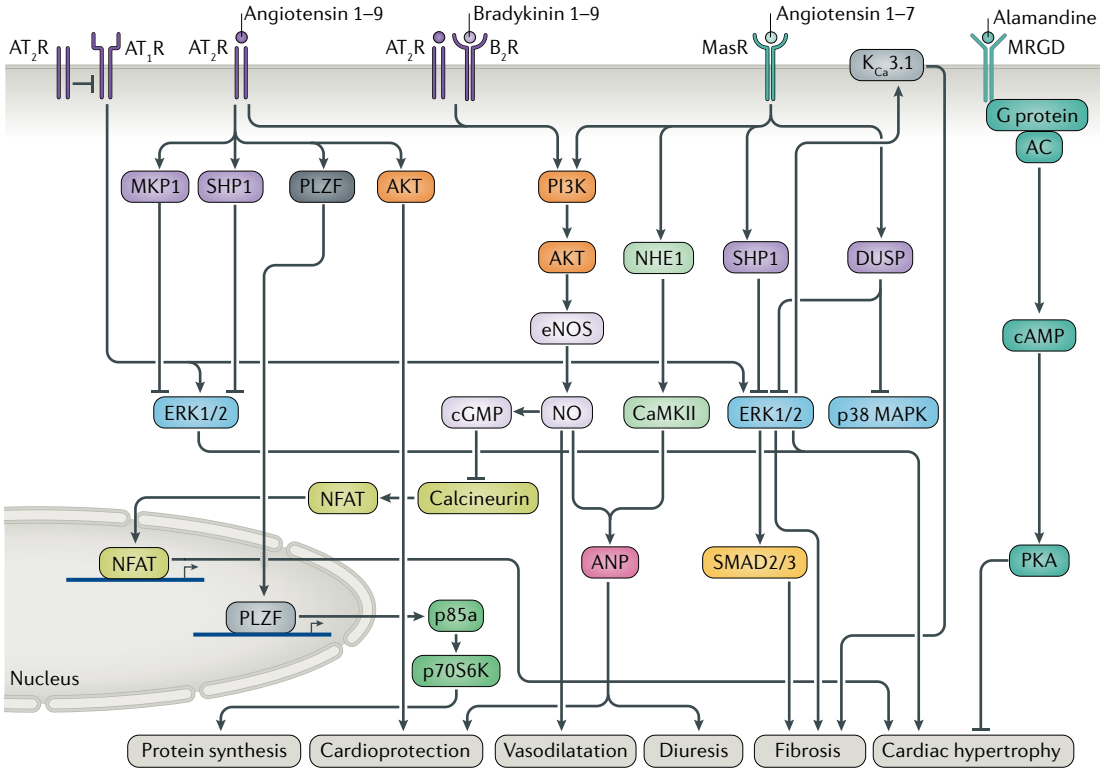


Fig. 3 | **Signal transduction mechanisms of the counter-regulatory RAS.** Signalling through the type 2 angiotensin II receptor (AT_2R) can directly inhibit AT_1R activation and thus antagonize the effects of angiotensin II[191]. Stimulation of AT_2R can also inhibit extracellular signal-regulated kinase 1 (ERK1) and ERK2 by activating Src homology region 2 domain-containing phosphatase 1 (SHP1)[192] and mitogen-activated protein kinase-phosphatase 1 (MKP1)[193], which can result in attenuation of cardiac hypertrophy. AT_2R can also activate the transcription factor promyelocytic zinc finger protein (PLZF), thereby inducing the expression of ribosomal protein S6 kinase β1 (p70S6K) and p85α expression and, in turn, eliciting protein synthesis[194]. In addition, AT_2R might trigger vasodilatation by activating the phosphatidylinositol-3-kinase (PI3K)–AKT–endothelial nitric oxide synthase (eNOS)–nitric oxide (NO)–cGMP pathway either via angiotensin 1–9-mediated activation[194–196] or by heterodimerization with bradykinin B_2 receptor (B_2R)[197]. Phosphorylation of AKT by activation of AT_2R through angiotensin 1–9 binding has also been found to confer cardioprotection[6]. Angiotensin 1–7 might induce the NO–soluble guanylyl cyclase pathway, thereby triggering vasodilatation via proto-oncogene Mas receptor (MasR) activation. Activation of this receptor can also reduce cardiac fibrosis by stimulating SHP1[198] and dual-specificity phosphatase (DUSP)[199], consequently inhibiting p38 mitogen-activated protein kinase (MAPK) and ERK1 and ERK2[200]. The $K_{Ca}3.1$ channel[201] and mothers against decantaplegic homologue 2 (SMAD2) and SMAD3[202] are downstream targets of ERK1 and ERK2, and are downregulated upon MasR activation. Additionally, angiotensin 1–7 exerts an anti-hypertrophic effect by inhibiting nuclear factor of activated T cells (NFAT) through a MasR–PI3K–AKT–NO–cGMP-dependent pathway[203]. This anti-hypertrophic effect also depends on atrial natriuretic peptide (ANP) secretion during atrial pacing and is associated with activation of the Na^+/H^+ exchanger (NHE1) and calcium/calmodulin-dependent protein kinase II (CaMKII) via the PI3K–AKT pathway[200]. Cardiac hypertrophy can also be reduced by activation of the Mas-related G protein-coupled receptor member D (MRGD) by alamandine via adenylate cyclase (AC)–cAMP–protein kinase A (PKA) signalling[10].

damage by diminishing the inflammatory response and attenuating right ventricular hypertrophy, as well as reducing vascular wall thickness and alveolar septum thickness[50]. The AT_2R agonist compound 21 (C21) has also been shown to inhibit cardiopulmonary fibrosis and right ventricular remodelling in a rat model of monocrotaline-induced PAH[49]. To date, only one preclinical study has assessed the effect of angiotensin 1–9 on PAH[48]. Adult rats with PAH treated with angiotensin 1–9 showed reduced right ventricular weight and systolic pressure, as well as diminished lung fibrosis, pulmonary arteriole thickness and endothelial damage compared with untreated controls. These effects were dependent on activation of the AT_2R but not the Mas receptor. Treatment with angiotensin 1–9 also reduced plasma levels of the pro-inflammatory markers tumour necrosis factor (TNF), CC-chemokine ligand 2 (CCL2; also known as MCP1), IL-1β and IL-6[48].

Systemic hypertension and remodelling

The ACE2–angiotensin 1–7 axis. Numerous preclinical studies have shown that stimulating ACE2 with synthetic activators (such as XNT[51] and diminazene aceturate (DIZE))[52], Mas receptor agonists such as AVE0991[53], CGEN-856S[54] and CGEN-857[54] and human recombinant ACE2[55] can reduce blood pressure and attenuate cardiovascular damage. However, others studies have not found an association between hypertension and

ACE2 activity. The synthetic ACE2-activator XNT reduced blood pressure in an angiotensin II-induced model of hypertension, but plasma concentrations of angiotensin II and angiotensin 1–7 remained unaltered[56]. Moreover, the antihypertensive effect of this drug was observed in ACE2-deficient mice, and neither XNT nor DIZE induced the enzymatic activity of ACE2 in rat or mouse kidneys[56]. These findings raise the question as to whether researchers should continue to focus on these drugs with unknown mechanisms of action. However, ACE2 remains an appealing therapeutic target for treating hypertension, especially in tissues in which expression of this enzyme is higher than in plasma[56]. The therapeutic potential of DIZE as an alternative treatment for hypertension and PAH has been shown in previous experimental studies[52,57]. Moreover, deoxycorticosterone acetate (DOCA)–salt hypertensive rats treated with the Mas receptor agonist AVE0991 had lower blood pressure levels than untreated controls[58]. The anti-hypertrophic effects of AVE0991 are, in part, mediated by inhibition of NADPH oxidase 2 and NADPH oxidase 4, as observed in hypertensive mice subjected to aortic banding[59]. At present, the effects of these ACE2 activators have only been evaluated in preclinical studies. A rigorous evaluation of how these agents exert their beneficial effects is needed before they can be tested in the clinical setting, in order to identify off-target and potentially toxic effects.

ACE2 activity has also been assessed in patients with high blood pressure. The level of ACE2-mediated angiotensin II-degrading activity in monocyte-derived macrophages in vitro has been found to be similar in cells from both healthy individuals and patients with hypertension[60]. Of note, ACE2 activity is significantly higher in monocyte-derived macrophages from patients with prehypertension than in those from patients with hypertension, suggesting a potential role for ACE2 as an early marker of hypertension. This finding might also indicate a physiological protective mechanism against hypertension, most probably through the rapid degradation of angiotensin II[60]. By contrast, no correlation has been found between hypertension and ACE2 activity in patients with ST-segment elevation myocardial infarction[61].

Plasma ACE2 levels have been suggested to vary depending on sex[62,63], although most of the research exploring the role of ACE2 in CVD has not considered sex-related differences in activity levels. During pregnancy, plasma levels of angiotensin II are significantly elevated, whereas angiotensin 1–7 levels are significantly diminished, which together might predispose pregnant women to hypertension-related complications[64]. Furthermore, levels of urinary angiotensin 1–7 in patients with hypertension have been reported to be inversely proportional to blood pressure levels, implying a crucial role for this peptide in the development of hypertension[65]. Finally, angiotensin 1–7 has also been shown to alleviate obesity-induced haemodynamic alterations[66].

Alamandine. Alamandine is a heptapeptide formed by the catalytic action of ACE2 on angiotensin A or directly from angiotensin 1–7 in the heart. Oral administration of an inclusion compound of alamandine and β-hydroxypropyl cyclodextrin reduced blood pressure in spontaneously hypertensive rats and diminished myocardial fibrosis in isoprenaline-treated rats[67]. This anti-hypertensive effect was shown to have two phases. Initially, mean arterial pressure and left ventricular systolic pressure increased briefly in an AT_1R-dependent manner, followed by a reduction in these parameters, which persisted throughout the rest of the infusion period. This anti-hypertensive effect was reversed by PD123319, an AT_2R antagonist[68]. Additionally, alamandine treatment mitigated vascular remodelling in mice subjected to transverse aortic constriction[69]. Additional studies are required to further our understanding of the complex regulation of alamandine, the cell signalling cascades it triggers, and its therapeutic implications for hypertension and other CVDs. The normal range of alamandine levels in both healthy individuals and patients with hypertension should be established to provide a better understanding of the effect of RAS inhibition on alamandine plasma concentrations in this clinical context.

AT_2R agonists. The vasodilatory effects of AT_2R activation have been demonstrated in mice lacking[70,71] or overexpressing this receptor[72]. Mice lacking the AT_2R showed an increased response to angiotensin II and significantly elevated blood pressure levels[70,71], whereas transgenic overexpression of the AT_2R in vascular smooth muscle cells of mice reduced angiotensin II-induced vasoconstriction[72]. The anti-hypertensive effects of the AT_2R-selective agonists CGP42112A and angiotensin 1–9 have also been evaluated[73,74]. CGP42112A-treated obese rats had reduced blood pressure levels compared with untreated rats, which was associated with an increase in urinary sodium excretion[74]. This agonist also decreased blood pressure levels in spontaneously hypertensive rats[75] and prevented endothelial cell migration mediated by vascular endothelial growth factor signalling[76].

The specific Rho kinase inhibitor fasudil significantly increased plasma levels of angiotensin 1–9 in both normotensive and hypertensive rats[77]. In addition, fasudil reduced blood pressure levels and aortic Rho kinase and ACE activity, whereas mRNA and protein levels of ACE2 were increased in plasma and the aortic wall[77]. Interestingly, another study showed an increase in ACE and angiotensin II levels in patients at high risk of acute pulmonary embolism compared with healthy volunteers[78]. Moreover, in a rat model of acute pulmonary embolism, RhoA–ROCK signalling mediated an imbalance in RAS vasoconstrictors, which was reversed with ROCK inhibitors or an ACE2 activator[78]. These findings further highlight the protective effects that ROCK inhibition can exert in the setting of hypertension, atherosclerosis and pathological cardiovascular remodelling.

In a study by Ocaranza and colleagues, administration of angiotensin 1–9 reduced blood pressure levels in hypertensive rats and attenuated myocardial damage by inhibiting the development of ventricular hypertrophy and fibrosis; importantly, these effects were mediated through AT_2R but not Mas receptor signalling[73]. However, in a separate study, gene delivery of angiotensin 1–9 with an adeno-associated virus (AAV) in mice subjected to coronary artery ligation completely restored

systolic blood pressure levels and cardiac output compared with sham-treated mice, but histological analysis revealed only mild effects on cardiac hypertrophy and fibrosis[79]. Notably, Ocaranza and colleagues only evaluated angiotensin 1–9 administration for 2 weeks[73], compared with the latter study that examined the effects of this peptide for 8 weeks[79]. The conflicting findings between these two studies suggest that the attenuation of myocardial damage might be transient and not sustained in the long term. However, the latter study did not measure plasma levels of angiotensin 1–9. AAV-mediated gene delivery of angiotensin 1–9 might not have produced a therapeutic concentration of the peptide in the blood that would be sufficient to protect the heart from adverse structural remodelling. A study that tested the anti-hypertensive actions of angiotensin 1–9 in stroke-prone spontaneously hypertensive rats also found no evidence of a protective effect[80], but this study used a dose of angiotensin 1–9 that was six times lower than that used by Ocaranza and colleagues[73]. Additional studies are warranted to explore the anti-hypertensive and anti-remodelling effects of angiotensin 1–9 administration and the implications of the plasma levels of this peptide on cardioprotection. Although the efficacy of angiotensin 1–9 administration has not been explored in the clinical setting, in patients with acute respiratory distress syndrome, higher angiotensin 1–9 levels in plasma were associated with reduced mortality, whereas increased plasma angiotensin I levels were associated with increased mortality[81].

Heart failure

ACE2 is critical for heart function[82], vasodilatation[83] and fluid balance[84]. *Ace2*$^{-/y}$ mutant mice have impaired contractility, increased expression of hypoxia markers and increased circulating levels of angiotensin II compared with control mice[82]. Furthermore, *Ace2*$^{-/y}$ mutant mice develop angiotensin II-mediated dilated cardiomyopathy that is characterized by an increase in markers of oxidative stress and inflammation, pathological hypertrophy and impaired left ventricular function[85]. Interestingly, plasma levels of the soluble form of ACE2 have been reported to be elevated in patients with HF and reduced ejection fraction, suggesting that sustained activation of the counter-regulatory RAS in HF might be a compensatory mechanism to attenuate cardiovascular dysfunction[86]. The mechanisms underlying HF with preserved ejection fraction (HFpEF) remain poorly defined, but the progression of this disease has been proposed to be linked to hypertension-induced cardiac remodelling[87]. Given the anti-hypertensive and anti-remodelling effects of the counter-regulatory RAS described thus far, this non-canonical signalling pathway might be a potential therapeutic target for the treatment of HFpEF. Angiotensin II infusion in wild-type mice resulted in increased blood pressure levels, myocardial hypertrophy, fibrosis and diastolic dysfunction; these effects were exacerbated in *Ace2*$^{-/y}$ mice[88]. Conversely, treatment of angiotensin II-infused wild-type mice with rhACE2 reduced angiotensin II-induced superoxide production and blunted the cardiac hypertrophic response, highlighting a possible protective role for this enzyme in HFpEF[88].

Other components of the non-canonical RAS pathway are also involved in HF. Mice deficient in the alamandine receptor MRGD have left ventricular remodelling and severe dysfunction, and present with pronounced dilated cardiomyopathy[89]. Furthermore, infusion of the AT_2R agonist C21 for 7 days in rats with HF induced by coronary artery ligation led to a reduction in noradrenaline excretion, as well as decreased renal sympathetic nerve activity[90]. Additionally, C21 administration increased baroreflex sensitivity, suggesting a protective role for this drug in the setting of HF.

Collectively, these findings support a role for various components of the counter-regulatory RAS in HF, both as potential biomarkers and therapeutic targets. Additional clinical studies are needed to determine the levels of ACE2, angiotensin 1–9 and angiotensin 1–7 in patients with HF.

Myocardial infarction

The role of non-canonical RAS signalling in the development of myocardial infarction has been described. *ACE2* mRNA levels are elevated in the setting of myocardial infarction[91], whereas loss of ACE2 can further exacerbate cardiac damage[92]. By the same token, *Ace2* overexpression has been shown to alleviate myocardial damage induced by ischaemia–reperfusion in rats[93]. Furthermore, administration of angiotensin 1–7 (added to the oligosaccharide hydroxypropyl β-cyclodextrin) in rats with myocardial infarction improved cardiac function and reduced infarct size by 50%[42,43]. Likewise, transgenic rats overexpressing a fusion protein that leads to a selective increase in angiotensin 1–7 levels were less susceptible to reperfusion-induced arrhythmias and isoproterenol-induced hypertrophy than wild-type rats[94].

The cardioprotective role of AT_2R in preventing post-ischaemic cardiac remodelling has been documented[95,96]. Mice lacking AT_2R have aggravated myocardial infarction-induced HF and reduced survival compared with sham-treated mice[97]. Correspondingly, transgenic mice overexpressing AT_2R showed improved left ventricular function after myocardial infarction[98], and similar results were observed in rats with cardiac-specific overexpression of AT_2R[99]. Administration of the AT_2R agonist C21 to rats subjected to coronary artery ligation significantly improved recovery of left ventricular function and reduced cardiac remodelling after myocardial infarction[100]. Delivery of angiotensin 1–9 with an AAV vector into mice after the induction of myocardial infarction resulted in a reduction in sudden cardiac death and improved left ventricular function compared with control mice[79]. Importantly, angiotensin 1–9 had a positive inotropic effect, achieved by increasing calcium-transient amplitude and contractility through a protein kinase A-dependent mechanism[79]. Using an ex vivo approach with isolated rat hearts subjected to global ischaemia and reperfusion, Mendoza-Torres and colleagues showed that angiotensin 1–9 infusion can also reduce infarct size and apoptotic and necrotic cell death, and improve left ventricular function in an AT_2R-dependent and AKT-dependent mechanism[6]. Together, these data suggest that angiotensin 1–7 and angiotensin 1–9

might be valuable pharmacological tools for the treatment of myocardial infarction, given their acute and long-term cardioprotective effects.

Inflammation

Inflammatory processes are central to the development and progression of CVDs such as atherosclerosis, hypertension, myocardial infarction and HF[101–105]. A link between inflammation and RAS has previously been observed. T cells have an endogenous RAS that can regulate T cell function, NADPH oxidase activity and superoxide production[106,107]. Natural killer cells have also been shown to express renin, angiotensinogen, ACE and AT_2R[107]. In line with these observations, the pro-inflammatory state is thought to upregulate RAS signalling in the setting of hypertension[108]. Interestingly, human monocytes also express ACE and ACE2 and can produce angiotensin 1–7 and angiotensin 1–9[109]. Taken together, these data suggest that the immune system might also be involved in regulating the non-canonical RAS.

Activation of the Mas receptor has been shown to promote anti-inflammatory effects[110]. Mice lacking this receptor have an exacerbated inflammatory reaction after treatment with lipopolysaccharides compared with wild-type mice[111]. Therefore, Mas receptor activation might be a valuable therapeutic target to counteract the pro-inflammatory processes that promote the development and progression of atherosclerosis[112,113]. Indeed, the Mas receptor agonist AVE0991 inhibits atherogenesis in *Apoe*$^{-/-}$ mice[114]. Moreover, long-term angiotensin 1–7 treatment confers both vasoprotection (by improving endothelial function) and atheroprotection (by reducing lesion progression) in *Apoe*$^{-/-}$ mice[115]. Consistent with these observations, angiotensin 1–7 can activate signalling pathways critical for the resolution of inflammatory processes involved in asthma[116].

In addition to the Mas receptor, AT_2R signalling has also been associated with the regulation of inflammation. The AT_2R agonist C21 dose-dependently attenuates lipopolysaccharide-induced TNF and IL-6 production, but increased production of the anti-inflammatory cytokine IL-10[117]. Consistent with these observations, a separate study showed that administration of C21 in prehypertensive, obese Zucker rats reduced plasma levels of TNF and IL-6, whereas coadministration with the AT_2R antagonist PD123319 decreased IL-10 levels in the kidneys[118]. Furthermore, in Wistar rats subjected to left coronary artery ligation, C21 treatment reduced the production of the pro-inflammatory cytokines IL-1β, IL-6 and IL-2 in an AT_2R-dependent manner, improved systolic and diastolic ventricular function, and reduced scar size[119]. Angiotensin 1–9 administration has also been shown to reduce cardiac and renal inflammation in a DOCA–salt model of hypertension in rats, but this effect was independent of AT_2R[120].

From bench to bedside

Research into the counter-regulatory RAS has resulted in the generation of a substantial amount of intellectual property related to its study and use. Currently, 184 patent applications associated with this system have been filed, most related to angiotensin 1–7 and its analogues, AT_2R, the Mas receptor, ACE2 and angiotensin 1–9. Only 76 patents are related to cardiovascular applications involving the control of arterial pressure, vascular remodelling, cardiac remodelling and HF. Furthermore, the robust evidence collated from large numbers of preclinical studies on the counter-regulatory RAS has also prompted the initiation of numerous clinical trials. At the time of this report, 15 clinical trials that involve interventions with counter-regulatory RAS molecules in CVDs were ongoing, including two studies designed to evaluate the safety of recombinant ACE2 and angiotensin 1–7 in treating thrombocytopenia[121,122]. A further nine trials aim to assess the use of ACE2 in the treatment of pulmonary hypertension[123,124] and the safety and use of angiotensin 1–7 in hypoxia, hypertension, HF and coronary artery bypass surgery[125–131]. Two trials investigating the use of angiotensin 1–7 to treat peripheral arterial disease and obesity-associated hypertension[132,133] are currently in the pre-recruitment phase.

Challenges in interpretation

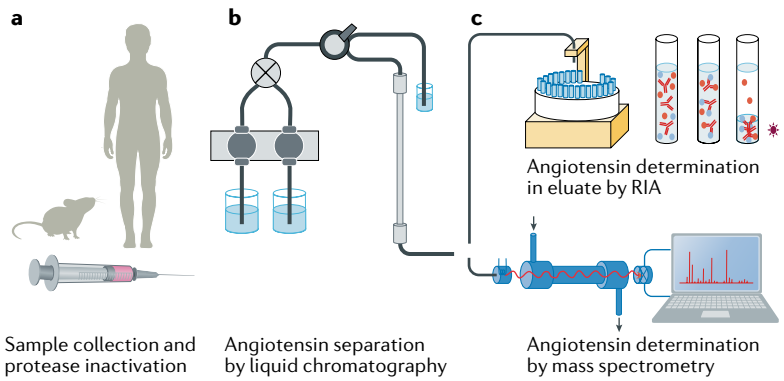
Despite the substantial amount of evidence suggesting a counter-regulatory role for the non-canonical RAS in protecting against the deleterious actions of a dysregulated classical RAS, the complexity of the relationship between the two systems remains to be fully elucidated. For example, ACE2 is elevated in patients with HF[86] or pre-hypertension[60], but depressed in patients with PAH[33]. These discrepancies suggest that the components of the counter-regulatory RAS are upregulated or downregulated depending on the stage, severity or type of CVD. Moreover, these conflicting findings reinforce our lack of knowledge of the physiological and pathophysiological mechanisms involved in non-canonical RAS regulation. For instance, elevated levels of soluble ACE2 might represent a compensatory mechanism in response to HF but might also be the result of increased cleavage of membrane ACE2 by disintegrin and metalloproteinase domain-containing protein 17, which is known to be upregulated in HF[86]. In addition, RAS peptides can also be modulated by pharmacological treatment. In this regard, patients with chronic HF treated with ACE inhibitors have elevated plasma levels of angiotensin 1–7 and reduced plasma levels of angiotensin II, whereas patients with acute HF treated with angiotensin II receptor antagonists have decreased plasma levels of angiotensin 1–7 and increased plasma levels of angiotensin II[134]. Furthermore, the addition of rhACE2 to plasma samples from patients with HF induced the conversion of angiotensin I and angiotensin II into angiotensin 1–9 and angiotensin 1–7, respectively[134].

Limitations in application

In addition to the aforementioned challenges in interpreting the data on the non-canonical RAS, the measurement of angiotensin 1–7, angiotensin 1–9 or alamandine in a clinical context poses many challenges. The separation of these peptides from a biological sample is difficult, given the similarity in their molecular structures. Angiotensin 1–7 is only two amino acids shorter than angiotensin 1–9[135], whereas angiotensin 1–7 and alamandine only differ in their N-terminal amino acid[67] (FIG. 2).

Box 2 | **Quantification of angiotensin peptides**

Three critical issues should be considered to ensure that the method for quantifying angiotensin peptides is reliable. First, the sampling procedure must be efficient because blood or tissue samples need to undergo immediate peptidase inhibition to ensure stabilization of angiotensin metabolites[157–162] (see the figure, part **a**). The sampling duration should be kept as short as possible to avoid unexpected shifts in angiotensin metabolite patterns. Second, the structural similarity of all angiotensin metabolites (FIG. 2) necessitates an effective separation procedure, usually liquid chromatography[157–162]. Once all peptides have been separated, angiotensin metabolites are quantified in the liquid chromatography eluate. Finally, the assay must be sensitive, given that angiotensin metabolites have been described at levels in the femtomolar range[157–162]. Immunological assays (radioimmunoassay (RIA) or enzyme-linked immunosorbent assays (ELISA))[157–160] and mass spectrometry[161,162] have been used in this setting. Both methods have lower limits of quantification for angiotensin metabolites (~1–2 fmol/ml in plasma and 5–10 fmol/g in tissue samples)[157–162]. Although RIA and ELISA are the traditional methods for quantifying angiotensin peptides given their high sensitivity and specificity, these methods rely heavily on the characteristics of the antibodies. Therefore, liquid chromatography–mass spectrometry is a promising approach for obtaining reliable read-outs, given its capacity to detect renin–angiotensin system (RAS) peptides by assessing their unique mass-to-charge spectra, which might also allow measurement of potential post-translational modifications in these peptides[163]. However, this technique is not without drawbacks. A complex spectrum resulting from measurement of multiple components with similar mass-to-charge ratios has been reported[164]. Moreover, this approach is expensive and requires very specific expertise[163]. These considerations are of paramount importance, given that inaccurate measurement of RAS peptides can lead to erroneous conclusions that might cloud our understanding of the non-canonical RAS.



Therefore, the identification of these peptides requires the use of high-precision approaches, such as high-performance liquid chromatography and mass spectrometry (BOX 2). Furthermore, one of the fundamental problems associated with the use of these peptides in the clinical context is their short plasma half-life, owing to rapid enzymatic degradation. In each of the numerous ongoing clinical trials assessing the effects of angiotensin 1–7 in CVDs, angiotensin 1–7 is administered via subcutaneous or intravenous injection[126–131]. However, a cyclized angiotensin 1–7 analogue has been described that has increased half-life, improved resistance to enzymatic degradation and superior functional activity compared with natural angiotensin 1–7[136]. Similar chemical modifications to the angiotensin 1–9 peptide might also prolong the half-life of the peptide. However, the non-peptide agonist C21, which has a half-life of 4–6 h, can also induce AT_2R activation[137]. This agonist has high selectivity for its receptor and is well tolerated[137,138]. However, although the results to date are promising, angiotensin 1–9 still requires extensive safety and efficacy assessment as a potential endogenous AT_2R agonist. The oral bioavailability of C21 is only 30%, and this agonist has also been reported to modulate epigenetic mechanisms associated with the pathophysiology of diabetic nephropathy[137], raising the possibility of unwanted off-target effects if used to treat CVD. Studies comparing the effects of C21 and angiotensin 1–9 will be useful to establish the potential differences between the two agents.

Complementary agents

Once the challenges hindering clinical translation of counter-regulatory RAS components for the treatment of CVD have been overcome, these therapeutic agents might be used to complement traditional pharmacological treatments. Such complementary drugs are necessary, because even gold-standard drugs for hypertension are associated with issues such as suboptimal drug efficacy and adherence. Most patients with hypertension, especially those with comorbidities, require two or more drugs to manage their blood pressure levels[139,140]. Furthermore, many of these patients require two or more doses each day[140], suggesting that the separate use of ACE inhibitors or angiotensin II receptor antagonists is not always effective. The use of more than one drug and the need for multiple doses per day can increase the incidence of adverse events, which can result in loss of adherence[140,141]. In addition, a longitudinal study that evaluated the dosing histories of 4,783 patients taking antihypertensive drugs found that nearly half of the patient cohort discontinued the treatment[142], which results in poorly controlled hypertension[143]. Combining these counter-regulatory RAS peptides with the current gold-standard antihypertensive drugs in one pill might overcome the need for patients with hypertension and other comorbidities to receive more than one drug or multiple dosages of drugs per day. Counter-regulatory RAS peptides, such as angiotensin 1–7, alamandine or angiotensin 1–9, have been found to be effective in reducing blood pressure and attenuating cardiovascular remodelling in preclinical studies[67,73,144]. These effects might be achieved with fewer adverse reactions in patients with hypertension compared with current antihypertensive therapies, which in turn might improve treatment adherence. Combining angiotensin 1–7 with the angiotensin-receptor blocker losartan might increase or extend its blood pressure-lowering capacity[145]. Importantly, the anti-atherosclerotic effects of dual angiotensin 1–7 and losartan therapy are synergistic[146]. Pharmacological synergy between current gold-standard treatment for CVDs and counter-regulatory RAS peptides might decrease the dosages required to achieve efficacy, thereby reducing adverse effects. However, although the endogenous origin of counter-regulatory RAS components suggests a safe pharmacological profile, the current lack of robust evidence in patients means that this hypothesis remains to be tested.

Conclusions

The evidence supporting the protective role of the counter-regulatory RAS in CVD is robust but incomplete. In addition to the methodological pitfalls that must be overcome, future research should also be

conducted in large animals with high translational value to further confirm the data from the studies carried out in vitro and in small-animal models. The roles of other RAS peptides, such as angiotensin III and angiotensin IV, in the cardiovascular system warrant further investigation. Furthermore, the assessment of classical and counter-regulatory RAS peptides during routine clinical evaluation in patients with CVD should be considered, although development of practical, affordable and accurate methods to assess these levels are required to achieve reliable readouts. The balance — or imbalance — of the levels of these peptides in plasma or urine might be useful as markers of CVD. Moreover, a thorough evaluation of the counter-regulatory RAS profile of each patient might bring current therapeutic approaches a step closer to the goal of precision medicine, allowing tailored treatment plans for each patient to optimize drug efficacy and adherence.

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All authors researched data for the article, contributed to discussion of the content and wrote, reviewed and edited the manuscript before submission.

Competing interests
M.P.O., M.C., J.E.J. and S.L. have patents related to the pharmacological effects of angiotensin 1–9. R.A.S.S. has patents related to the pharmacological effects of angiotensin 1–7 and alamandine. J.A.R. and L.G. declare no competing interests.

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