Angiotensin-(1-7) and angiotensin-(1-9): function in cardiac and vascular remodeling

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Summary

The renin angiotensin system (RAS) is integral to cardiovascular physiology, however, dysregulation of this system largely contributes to the pathophysiology of cardiovascular disease (CVD). It is well established that angiotensin II (Ang II), the main effector of the RAS, engages the angiotensin type 1 receptor and promotes cell growth, proliferation, migration and oxidative stress, all processes which contribute to remodeling of the heart and vasculature, ultimately leading to the development and progression of various CVDs including heart failure and atherosclerosis. The counter-regulatory axis of the RAS, which is centered on the actions of angiotensin converting enzyme 2 (ACE2) and the resultant production of angiotensin-(1-7) (Ang-(1-7) from Ang II, antagonizes the actions of Ang II via the receptor Mas, thereby providing a protective role in CVD. More recently, another ACE2 metabolite, Ang-(1-9), has been reported to be a biologically active peptide within the counter-regulatory axis of the RAS. This review will discuss the role of the counter-regulatory RAS peptides, Ang-(1-7) and Ang-(1-9) in the cardiovascular system, with a focus on their effects in remodeling of the heart and vasculature.

Introduction

The renin angiotensin system

The renin angiotensin system (RAS) is a key component of cardiovascular physiology playing an integral role in the regulation of vascular tone, blood pressure and electrolyte balance (Figure 1). However, dysregulation of the RAS largely contributes to the pathogenesis of various cardiovascular diseases (CVD). The extent to which chronic dysregulation of the RAS contributes to CVD is demonstrated by the fact that inhibitors of this system, including angiotensin converting enzyme (ACE) inhibitors and angiotensin type 1 receptor (AT_1R) antagonists are among the most effective treatments [1]. Angiotensin converting enzyme (ACE), which is most highly expressed in the vascular endothelium, lung, kidneys and intestine, converts Ang I to angiotensin II (Ang II), the main effector of this system [2-4]. Ang II is integrally involved in regulation of vascular tone, blood pressure and electrolyte balance. The effects of Ang II are mediated via interaction with two G protein coupled receptors (GPCRs) the angiotensin type 1 and type 2 receptors (AT_1R and AT_2R, respectively), which tend to have opposing actions, however the majority of Ang II's effects are mediated via the AT_1R (Figure 1). Ang II stimulation of the AT_1R results in coupling of the receptor to various heterotrimeric G-proteins including $G_{q/11}$, $G_{i/o}$, and $G_{12/13}$ resulting in multiple signaling cascades leading to vasoconstriction, proliferation, sodium retention, and oxidative stress, all processes which are largely involved in the pathogenesis of CVD (reviewed in [5]). In contrast to the AT_1R, the role and function of the AT_2R is not as clearly defined. However, it is thought to counterbalance some actions of the AT_1R by inhibiting growth and proliferation, reducing tissue remodeling, and increasing vasodilation and nitric oxide (NO) production [6-10].

The counter regulatory axis of the renin angiotensin system

The traditional view of the RAS has undergone significant changes, largely due to the discovery of the enzyme ACE2 [11-13] (Figure 1). ACE2 is a homologue that shares about 42% identity with ACE and is highly expressed in a number of tissues including the heart, vasculature and

kidney [12-13]. Due to structural differences ACE acts mainly as a peptidyl dipeptidase, while ACE2 acts as a carboxypeptidase [11]. ACE2 functions in the RAS by cleaving the C terminal residues from Ang I and Ang II, thereby reducing Ang II levels (and hence reducing its deleterious effects) and producing angiotensin-(1-9) [Ang-(1-9)] and angiotensin-(1-7) [Ang-(1-7)], respectively [13]. Ang-(1-9) can be further cleaved by ACE to increase Ang-(1-7) levels [13] (Figure 1).

The majority of Ang-(1-7) is produced via the actions of ACE2 and it is its main product as ACE2 has approximately 400-fold greater affinity for Ang II than Ang I [14]. Ang-(1-7) can also be produced less efficiently via hydrolysis of Ang-(1-9) by ACE or via the actions of alternative enzymes including prolyl endopeptidase, neutral endopeptidase or thimet oligopeptidase [13, 15-16](Figure 1). The half life of Ang-(1-7) in the circulation is approximately 10 seconds [17-18] and circulating levels of Ang-(1-7) are reported to be 20 pg/mL [19]. Ang-(1-7) can oppose the actions of Ang II in a number of tissues, mainly by inhibiting cell growth, migration, and inflammation that occurs as a result of Ang II, ultimately preventing adverse remodeling and subsequent dysfunction of the cardiovascular system [20-23]. Originally the mechanism by which Ang-(1-7) exerted its effects was unknown but research by Rowe *et al* (1995) suggested that due to low affinity for both angiotensin receptors, the effects of Ang-(1-7) were unlikely to be mediated by signaling via either of the angiotensin receptors [24]. Receptor binding studies later showed that Ang-(1-7) could bind to the G-protein coupled receptor Mas and further research in Mas-deficient mice identified Ang-(1-7) as the endogenous ligand for this receptor [25]. Moreover, despite the wealth of evidence that indicates Ang-(1-7) is a Mas ligand, there is also more recent evidence that while Ang-(1-7) has relatively low affinity for the AT_2R, it may also elicit certain biological effects via this receptor. For example, in stable cell lines generated to express either AT_1R or AT_2R, Ang-(1-7) was found to bind the AT_2R with higher affinity than the AT_1R [26]. The Ang-(1-7)/AT_2R interaction has also been observed *in vivo*. In isolated mouse hearts exposed to the AT_2R antagonist PD123, 319, Ang-(1-7) increased perfusion pressure, an effect not observed with Ang-(1-7) infusion alone and which was independent of both the AT_1R or Mas[27]. It was also observed that in the presence of AT_1R blockade, Ang-(1-7) reduced blood pressure in both normotensive and spontaneously hypertensive stroke prone rats (SHRSP), an effect mediated via the AT_2R [28]. This effect was preserved in aged normotensive rats under similar experimental conditions, however, the vasodepressor effect was via both the AT_2R and Mas receptor in the aged rats [29]. Conversely, in mice lacking the angiotensin receptors, Ang-(1-7) reduced mean arterial pressure, suggesting that the AT_2R may not be responsible for the vasodepressor effect of Ang-(1-7) [30].

In contrast to Ang-(1-7) there is much less known about Ang-(1-9). Ang-(1-9) can be generated from Ang I by ACE2 [13] or through the activity of carboxypeptidase A or cathepsin A[31-32]. While circulating levels of Ang-(1-9) have been reported to be around 2-6 fmol/mL in healthy subjects, these levels are thought to increase in pathological states [32-34]. For example, in human heart failure patients, Ang-(1-9) is formed at a rate of 1nM/min/mg in the myocardium and a large proportion of available Ang I is rapidly converted to equal levels of Ang-(1-9) and Ang II, suggesting that in pathological conditions the heart functions to increase levels of Ang-(1-9) [32]. However, there is currently little known about the biological effects of Ang-(1-9) in

the cardiovascular system. Originally it was thought to be biologically inactive, contributing indirectly to counteregulate actions of Ang II by competing with Ang II for the ACE enzyme active site, resulting in reduced Ang II and increased Ang-(1-7) level [13, 35]. However, recent research has demonstrated that Ang-(1-9) exerts direct biological effects in the cardiovascular system, and these effects may be via the AT_2R [36]. Using radioligand binding assays it was demonstrated that Ang-(1-9) could bind to both the AT_1R and AT_2R [36] and in cardiomyocytes Ang-(1-9) mediated anti-hypertrophic effects via the AT_2R as PD123,319 (an AT_2R antagonist) blocked these effects [36]. This suggests that despite having approximately 100 fold lower affinity for the AT_2R than Ang II, Ang-(1-9) may elicit functional effects via this receptor (Figure 1-2). While further work is required to fully establish the signaling mechanisms elicted by Ang-(1-9), the selective activity of this peptide at the AT_2R is possibly due to the pharmacological concept of functional selectivity where ligands have the ability to induce unique, ligand specific conformations that can result in differential activation of signalling pathways [37]. It has previously been shown that while the AT_1R exists in a constrained conformation, the AT_2R exists in a relaxed state [38] and it is therefore has been postulated that the additional histidine present in Ang-(1-9) may stabilise the AT_2R in a distinct conformational state, leading to the activation of pathways to counter-regulate the effects of AngII and the AT_1R, however, this remains to be demonstrated experimentally [36]. Alternatively, Ang-(1-9) may be metabolized Ang-(1-7) or another peptide, which may act at either at the AT_2R with high affinity or an alternative receptor which is also sensitive to PD123, 319, as has previously been shown for the recently reported counter-regulatory RAS receptor, Mas related gene D receptor (MrgD) [39].

The counter regulatory axis of the RAS in cardiac remodeling

Ang II signaling via the AT_1R is a key mechanism which drives changes in cardiac structure and function, known as remodeling, post-cardiac injury *in vivo* which can lead to the progressive decline of cardiac function. Initially, remodeling within the heart is an adaptive process where changes to the myocardium which are required for maintenance of cardiac function in response to injury occur. However, over time this remodeling becomes maladaptive, leading to chronic remodeling that is associated with development of cardiac dysfunction and increased risk of heart failure [40]. These changes affect both cardiac structure and function and include cardiac hypertrophy, fibrosis and dilation; inflammation and secondary electrical remodeling which causes changes in various ion channels and excitation-contraction coupling (ECC) [40-44]. Both Ang-(1-7) and Ang-(1-9) have been demonstrated to mediate a beneficial role in cardiac structural and functional remodeling and this evidence is detailed in the following sections. In addition to a direct protective effect of the peptides, modulation of levels of the angiotensin peptides by ACE2 has been shown to be beneficial. Various studies using ACE2 knockout mice have demonstrated that loss of this enzyme results in increased Ang II levels in the heart, kidneys and plasma, associated with impaired cardiac function and increased remodelling following myocardial infarction [45-46]. Furthermore, diabetic Akita mice deficient in ACE2 expression develop cardiac myopathy, associated with increased Ang II levels and AT_1R signaling [47]. These studies suggest a protective role for ACE2 in cardiac remodelling through reduced Ang II production. However, other studies have shown the opposite in that overexpression of

ACE2 is potentially associated with a worsened cardiac phenotype [48-49]. This phenomenon could be due to supraphysiological levels of expression at specific unwated sites and therefore further studies are required to address this conflicting data.

Structural remodeling of the heart

Effects of angiotensin-(1-7)

Ang-(1-7) has been extensively studied with regards to its effects in the heart (Figure 2). Collagen deposition in the spontaneously hypertensive rat (SHR) heart as a result of salt-induced cardiac remodeling has been shown to be associated with decreased levels of cardiac Ang-(1-7) and Mas, independent of changes in cardiac Ang II levels, suggesting the loss of the protective effect of Ang-(1-7) contributes to hypertension-induced fibrosis in this model [50]. Lack of Mas was also shown to increase levels of collagens type I, type III and fibronectin in the hearts of Mas-deficient mice [51]. In the rat deoxycorticosterone acetate (DOCA)-salt model of hypertension, Ang-(1-7) delivered via minipump was shown to prevent myocardial and perivascular fibrosis, which was shown to be independent of effects on blood pressure or cardiac hypertrophy [52]. Using a transgenic rat strain TG(hA1-7)L7301, which expresses an Ang-(1-7) fusion protein in a cardiac-specific manner via the alpha-myosin heavy chain (α-MHC) promoter demonstrated that the transgenic rats were more resistant to isoproterenol-induced cardiac stress than wild type controls [53]. After high-dose isoproterenol administration for seven days, analysis of collagen I, III and fibronectin showed reduced deposition in the heart compared to control rats [53]. Furthermore, in the TGR(A1-7)3292 rats higher circulating levels of Ang-(1-7) were also apparent and the rats were particularly resistant to isoproterenol-induced cardiac hypertrophy [54]. A further study, utilizing transgenic TGVII-7 mice over-expressing a cardiac-specific Ang-(1-7) fusion protein under control of the mouse α-MHC promoter also reported reduced cardiac remodeling in response to Ang II infusion via osmotic minipump, with reductions in both fibrosis and hypertrophy [55]. Protective cardiac effects have also been observed following gene transfer of Ang-(1-7). In a study performed in a rat model of myocardial infarction (MI), employing lentiviral delivery of Ang-(1-7) via direct injection to the left ventricle followed by ligation of the left anterior descending coronary artery (LAD), it was observed that Ang-(1-7) was able to attenuate cardiac hypertrophy and reduce left ventricular wall thinning [56]. Mechanistically, it was suggested that the peptide may exert beneficial effects through modulation of ACE2 and the bradykinin B_2R, which were both found to be up-regulated in the myocardium of Ang-(1-7) infused animals [56]. Furthermore, these findings indicated that Ang-(1-7) may also have anti-inflammatory properties as reduced levels of the pro inflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), but increased expression of the anti-inflammatory cytokine interleukin-10 (IL-10) were observed [56].

In vitro studies in both adult and neonatal rat cardiac fibroblasts have demonstrated that Ang-(1-7) inhibits proliferation and collagen production [57-58]. In neonatal rat cardiac fibroblasts Ang-(1-7) achieved this via reduced Ang II-mediated phospho-ERK1 and –ERK-2 production and

increasing dual specificity phosphatase 1 (DUSP1) activity, which in turn reduced MAP-kinase activity and prevented production of pro-proliferative prostaglandin-2 [58]. Further work studying cardiac remodeling *in vivo* has identified a role for the ERK and protein-kinase pathways in the actions of Ang-(1-7). In the SHR it has been demonstrated that restoration of abnormal ACE2 in the heart results in an increase in cardiac Ang-(1-7) production, which in turn leads to reduced ventricular collagen content [59]. This was shown to be associated with a decrease in phosphorylation of ERKs which are known to participate in cardiac fibroblast collagen synthesis[59]. In an Ang II infusion model of cardiac remodeling in ACE2 knockout mice infused with recombinant human ACE2 (rhACE2) mice showed a significant reduction in multiple cardiac remodeling processes and this was in an Ang-(1-7)-dependent manner [60]. Again, Ang-(1-7) mediated reduced collagen and fibronectin production and attenuated cardiac hypertrophy. Furthermore, using rhACE2 infusion, and thereby raising Ang-(1-7) levels in a wild type mouse model of pressure-overload induced cardiac remodeling, partial attenuation of the development of dilated cardiomyopathy was reported [60]. This was demonstrated to be via inhibition of mitogen-activated JAK2-STAT3 and PKC activity, as well as diminished Ang II mediated ERK1/2 activation [60].

As well as cardioprotective effects with regards to fibrosis and hypertrophy, Ang-(1-7) has recently been shown to promote angiogenesis in infarcted rat hearts in a Mas-dependent manner via up-regulation of vascular endothelial growth factor D (VEGF-D) and matrix metalloproteinase-9 (MMP-9) [61]. Ang-(1-7) has also been demonstrated to reduce production of cardiac reactive oxygen species in response to cardiac injury as well as increased nitric oxide (NO) production, thus further providing a cardioprotective effect [60]. Ang-(1-7) infusion in the SHR was shown to increase expression levels of ventricular endothelial and neuronal nitric oxide synthase (eNOS and nNOS, respectively), as well as an increase in NOS phosphorylation and activity [62]. These effects were via AT_2R and bradykinin B_2R dependent pathways, as demonstrated using the receptor antagonists PD123,319 and icantibant, respectively, as opposed to via the Mas axis, suggesting that some effects of Ang-(1-7) may be mediated via cross-talk between the AT_2R and BK_2R [62]. The effect of Ang-(1-7) on oxidative stress was further demonstrated using chimeric mice that mimic human RAS activation[63]. These mice were generated by crossing human renin (*hRN*) and human angiotensin (*hANG*) transgenic mice which were then utilized as a mouse model of human hypertension [63]. These mice demonstrated that depletion of Ang-(1-7) via reduced ACE2 activity exacerbated hypertension-induced cardiac remodeling. In chimeric mice, L-NAME treatment in order to persistently inhibit NO production resulted in an increase in blood pressure compared to wild-type mice, and was accompanied by accelerated oxidative-stress induced cardiovascular remodelling as well as reduced cardiac ACE2 expression[63]. Following AT_1R antagonist (olmesartan) treatment, L-NAME induced remodeling and cardiac ACE2 reduction was prevented, and this effect was reversed by co-administration of the Ang-(1-7) antagonist A779 . A characteristic of these animals is increased NADPH oxidase production which results in a rise in reactive oxygen species (ROS) in the myocardium as a result of remodeling. Use of the AT_1R antagonist losartan reduced ROS levels through inhibition of Ang II actions and in a partially Ang-(1-7) dependent manner.

The vast array of studies looking at the effect of Ang-(1-7) on structural remodeling has shown that it is an important regulator in remodeling processes and key in counteracting Ang II signaling, identifying it as a potential therapy for human cardiac remodeling.

Effects of angiotensin-(1-9)

The first evidence to suggest that Ang-(1-9) elicits independent biological effects in the heart was reported by Ocaranza *et al* (2006), using a rat coronary artery ligation model of MI, where cardiac hypertrophy and dysfunction are present at 8 weeks, and in which they assessed peptide and enzyme levels [34]. It was found that 1 week post-MI, circulating levels of Ang-(1-9), along with levels of Ang II, ACE, and ACE2, were increased compared to control animals, however at 8 weeks only Ang II and ACE remained high, with circulating levels of ACE2 and Ang-(1-9) diminishing to levels lower than the control group [34]. Treatment of the animals with the ACE inhibitor enalapril prevented the changes observed at 8 weeks, suggesting generation of Ang-(1-9) via ACE2 is able to counter-regulate Ang II-mediated actions. Circulating Ang-(1-7) levels were reported to be unchanged throughout the study [37]. A later study demonstrated an antihypertrophic role for Ang-(1-9) *in vitro* using neonatal cardiac myocytes and *in vivo* in the rat model of MI. Importantly, it was demonstrated that these actions were independent of the Ang-(1-7) receptor Mas using simultaneous delivery of the Mas receptor antagonist, A779 [64].

Further *in vitro* studies by Flores-Munoz *et al* (2011) established an antihypertrophic role for Ang-(1-9) in primary adult rabbit cardiomyocytes and rat neonatal H9c2 cardiomyocytes stimulated with Ang II [36]. This study was first to provide a direct comparison between Ang-(1-9) and Ang-(1-7). Importantly the effect of Ang-(1-9) was selectively inhibited by the AT_2R antagonist PD123,319, while those of Ang-(-1-7) were only inhibited in the presence of the Mas antagonist A779, demonstrating for the first time a direct, receptor mediated biological action for Ang-(1-9) [36]. Intriguingly, use of PD123,319 had no effect on the effects of Ang II, suggesting that in this experimental setting selective Ang-(1-9) functional activity at the AT_2R is important. The effects of Ang-(1-7) were only blocked by the Mas antagonist A779 and not the AT_2R antagonist PD123,319 despite previous studies suggesting a role for Ang-(1-7) mediating effects via the AT_2R [28]. Clearly this requires further study and it may be that the effects of individual angiotensin peptides at the angiotensin receptors are tissue and/or pathology specific. Ang-(1-9) was further studied via peptide infusion *in vivo* and demonstrated similar benefits to Ang-(1-7) in cardiac structural remodeling, but through what appears to be an independent mechanism (Figure 2) [65]. In the SHRSP osmotic minipump mediated infusion of Ang-(1-9) for 4 weeks resulted in a 50% reduction in cardiac fibrosis compared to control animals [65]. This was demonstrated to be AT_2R-dependent via infusion of the antagonist PD123 319 which attenuated the anti-fibrotic effects of the peptide. Unlike Ang-(1-7), very little is known about the potential mechanisms through which Ang-(1-9) may act as signaling via the AT_2R is currently poorly characterized. Activation of various pathways that involve tyrosine or serine/threonine phosphatases have been suggested as potential signalling mechanisms[66]. Phosphatases suggested to be involved in AT_2R signalling are mitogen-activated protein kinases

phosphatases 1 (MKP-1), SH2 domain containing phosphatases (SHP-1) and protein phosphatases 2A (PP2A) [67]. Interestingly, these phosphatases have been shown to interact with the ERK1/2 pathway [68], which is known to regulate collagen synthesis, fibroblast proliferation and cardiac hypertrophy. Therefore, further research is required to fully delineate the mechanism of action of Ang-(1-9) in the heart. Additionally, alternative approaches to assess the role of the AT_2R for Ang-(1-9) are required since the pharmacological antagonist PD123,319 has also been reported to be a competitive antagonist at the recently identified counter-regulatory RAS receptor, Mrg D receptor [39].

Functional and electrical remodeling in the heart

Structural remodeling changes are accompanied by detrimental changes in cardiac function and contractility. Fibrosis alters the mechanical properties of the heart, leading to decreased compliance of the ventricle and impedance and desynchronization of electrical conductance through the heart. Eventually this results in diastolic dysfunction, followed by contractile dysfunction, leading to an increased risk of arrhythmia [44, 69]. ECC, where electrical excitation of cardiomyocytes drives contraction of the heart, is also affected by Ang II remodeling processes, with alterations in the calcium sensitivity of calcium handling proteins and changes in ion current expression in cardiomyocytes, leading to altered intracellular calcium concentrations which in turn may reduce cardiac contractility [70].

Studies on the effect of Ang-(1-7) in models of cardiac dysfunction have demonstrated the potential of the peptide to improve or maintain cardiac contractility and that it exerts anti-arrhythmogenic properties. Lentiviral-mediated delivery of Ang-(1-7) directly to the left ventricle of rat hearts which were then subject to MI by ligation of the LAD not only prevented structural changes but also prevented decline in cardiac function [56]. Electrocardiography (ECG) and hemodynamic measurements demonstrated improvements in fractional shortening and left ventricular systolic pressure and attenuation of rises in left ventricular diastolic pressure. The beneficial functional effects of Ang-(1-7) were also shown using peptide infusion in a rat heart failure model, where restoration of left ventricular systolic and diastolic pressure was observed in peptide infused animals [71]. In isolated cardiomyocytes from transgenic TGR[A1-7]3292 rats Ang-(1-7) preserved the calcium transient compared to cardiomyocytes isolated from control Ang II infused rats, which presented a 26 % reduction in intracellular calcium amplitude[72]. This is of note as changes in expression levels of cardiomyocyte calcium handling proteins and especially alterations in intracellular and sarcoplasmic reticulum calcium concentrations are prominent features of contractile dysfunction and development of heart failure [41, 72]. Moreover, this study went on to identify possible mechanisms by which Ang-(1-7) exerts these effects, suggesting a role for nitric oxide/guanosine 3,5-cyclic monophosphate in regulating the cardioprotective effects observed [72]. A similar study in the TG(hA1-7)L7301 rat also demonstrated enhancement of single cardiomyocyte calcium handling due to cardiac overexpression of Ang-(1-7) [53]. Cardiomyocytes from these animals exhibited increased calcium transient amplitude, increased rate of transient decay and increased expression of sarcoendoplasmic reticulum calcium transport ATPase 2a (SERCA2a). Furthermore, isolated

hearts from these rats also showed a reduced rate of ischemia/reperfusion injury arrhythmias, suggesting Ang-(1-7) can act directly on the heart to improve cardiac function [53]. Beneficial functional effects of Ang-(1-7) were also seen in a study by Patel *et al* (2012), in ACE2 knock-out mice in pressure-overload induced heart failure[73]. Ang-(1-7) infused animals showed normalisation of fractional shortening, LV end diastolic pressures and $\pm dP/dt_{max}$ [73]. This was accompanied by normalisation of heart weight and hypertrophy marker expression levels as well as attenuated NADPH oxidase activation. Moreover, the benefits observed were comparable to those seen in animals treated with the AT_1R blocker irbesartan [73].

Many studies of Ang-(1-7) on electrical properties of the heart have been performed using *ex vivo* whole heart Langendorff preparations, where anti-arrhythmogenic effects in ischemia/reperfusion injury have been clearly demonstrated [54, 74-76]. Ang-(1-7) delivered via osmotic minipump in a dog pacing model of atrial fibrillation (AF) reduced interstitial fibrosis and as a result showed reduced susceptibility to and duration of induced AF [77]. Again this effect was believed to be modulated by reduction of ERK1 and ERK2 signaling [77]. In this model Ang-(1-7) also attenuated the decrease in action potential duration, characteristically observed in atrial myocytes during AF, as well as preventing the decrease in expression of the L-type calcium channel (I_{CaL}) and outward potassium channel (I_{TO}) observed in the model, however the mechanism by which this occurs remains to be clarified [77]. This demonstrates that Ang-(1-7) not only has potential benefits on cardiac function through anti-structural remodeling, but also that it has the potential to alter ion channel and calcium handling protein expression resulting in modulation of cardiac function. However, *in vitro* treatment of isolated adult mouse cardiomyocytes with 10 nM Ang-(1-7) has no direct effect on calcium handling properties, whereas conversely, absence of Mas in $Mas^{-/-}$ mouse cardiomyocytes results in a reduced calcium transient peak and slower calcium transient kinetics [78]. This suggests more work is needed to elucidate the role of Ang-(1-7) in modulating cardiac function. The requirement for further work is highlighted by recent excting findings regarding Ang-(1-7). Ang-(1-7) infusion in a murine MI model was recently demonstrated to improve cardiac function via stimulation of circulating and bone marrow derived endothelial progenitor cells (EPC), leading to enhanced EPC migration to the heart [79]. Therefore, this provides evidence that therapeutic effects of Ang-(1-7) may also extend to enhancing regenerative cell therapy. These findings have been extended to show that lentiviral over-expression of ACE2 in murine EPCs enhanced their migration and ability to form tubes in vitro [80]. Clinically, isolation of CD34+ cells from the blood of diabetes patients demonstrated that their nitric oxide bioavailability and migratory and proliferative function could be improved via exposure to Ang-(1-7) [81]. Furthermore, transduction of the EPCs with a lentiviral vector expressing Ang-(1-7) enhanced the reparative ability of the cells in a murine model of diabetic retinopathy. As a point of note in cancer clinical trials Ang-(1-7) infusion has been demonstrated to mediate complete restoration of haematopoietic cell profile following chemotherapy in patients, highlighting vast potential for this peptide in improving haematopoeitic and immune function [82].

There is currently very little reported evidence to suggest a beneficial functional effect of Ang-(1-9) in models of cardiac dysfunction. Chronic infusion of Ang-(1-9) has been demonstrated by echocardiography to prevent left ventricular wall thickening and left ventricular end systolic

and diastolic volumes (LVESV and LVEDV) in comparison to the MI [64]. However, there was no change observed in cardiac function between MI and Ang-(1-9) infused MI animals as measured via left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS).

Vascular remodeling

Vascular remodeling, characterized by changes in the size and composition of adult blood vessels, is an adaptive process that allows vessels to respond to changes in hemodynamic conditions but also underlies the pathogenesis of various major CVDs such as atherosclerosis. Damage or injury to the vessel wall results in denudation of the endothelial cell layer and reduced NO bioavailability [83]. It is this damage to the endothelium that predisposes to vascular remodeling by increasing the occurrence of thrombosis, creating an inflammatory environment at the site of injury and promoting growth and migration of vascular smooth muscle cells (VSMC), and degradation and reorganisation of the extracellular matrix [84-89]. Dysregulation of the RAS is one of the key contributing factors to remodeling of the vasculature and the development of atherosclerosis, with the majority of the pathological processes described above being mediated by Ang II signaling at the AT_1R [90]. In addition to its protective effects in the heart, the components of the counter-regulatory axis of the RAS have been shown to play an important role in the vasculature by inhibiting the pathological actions of Ang II (Figure 3). In atherosclerotic human carotid arteries, ACE2 activity was found to be increased in early stage atherosclerosis or in unstable lesions in comparison to stable lesions, demonstrating differential activity of ACE2 at different stages of the disease [91]. While it is currently unclear how this differential activity is regulated, it may be that ACE2 is increased in the early stages of atherosclerosis and in unstable lesions in an attempt to control the disease pathology and lessen injury by reducing Ang II production and increasing production of Ang-(1-7). Additionally, over-expression of ACE2 using adenoviral vectors has been shown to reduce atherosclerotic lesion size in $ApoE^{-/-}$ mice [22]. Moreover, ACE2 overexpression also stabilizes existing atherosclerotic lesions, inhibiting progression of lesion development at early stages of atherosclerosis, but not at advanced stages of the disease [92]. These effects were associated with both reduced levels of Ang II and increased levels of Ang-(1-7), suggesting a key role for ACE2 in balancing the activity of both axes of the RAS in atherosclerosis.

Effect of angiotensin-(1-7) in vascular remodeling

To date a large proportion of the protective effects of the counter-regulatory axis of the RAS in the vasculature have been attributed to the production of Ang-(1-7), and its resulting interaction with Mas (Figure 3). Numerous *in vitro* studies have shown that Ang-(1-7) signaling via Mas inhibits MAPK signaling pathways, mainly via reduced activity and expression of ERK1/2, leading to a reduction in VSMC proliferation [93] and migration [94]. The Ang-(1-7)/Mas interaction has also been shown to reduce VSMC proliferation and migration via release

of prostaglandins including prostacyclin (PGI2) and prostaglandin E2 (PGE2), resulting in increased cyclic adenosine monophosphate (cAMP) levels and inhibition of cyclo-oxygenases [95].

The anti-proliferative and anti-migratory role of Ang-(1-7) is also observed in numerous rodent models of vascular disease. Ang-(1-7), via Mas, reduces neointimal formation following balloon injury [96], stent implantation in rats [21], and angioplasty in rabbits [97], and has been associated with reduced atherosclerotic lesion size and neointimal formation following vascular injury in Apo $E^{-/-}$ mice [22, 98-99]. Additionally, a non-peptide agonist of Mas, AVE0991, which mimics the actions of Ang-(1-7) [100], inhibits rat VSMC proliferation *in vitro* [101].

Ang-(1-7) increases NO release, thereby acting as a vasodilator and improving vascular endothelial function [102-103]. Increase of NO is achieved directly via Mas-mediated stimulation of eNOS and sustained Akt phosphorylation, or indirectly via production of bradykinin and receptor cross talk with the bradykinin BK_2R [31, 104]. Additionally, Ang-(1-7) has been shown to promote vasodilation via the AT_2R in the SHRSP during AT_1R blockade[28]. This was shown to involve interaction between the AT_2R and BK_2R as vasodilation in response to Ang-(1-7) was prevented by both PD123,319 and HOE 140 (a BK_2R antagonist) [28].

An Ang-(1-7)-mediated increase in NO bioavailability has also been linked to reduced ROS production, thereby promoting improved vascular function and reduced atherosclerosis[105]. In Apo $E^{-/-}$ knockout mice chronic administration of Ang-(1-7) via osmotic mini pump restored renal endothelial function which was associated with increased NO bioavaliability [105]. To investigate the relationship between ROS levels and NO bioavailability in this setting, the ROS scavenger Tempol was used. While Tempol improved endothelial function in untreated Apo $E^{-/-}$, it had no effect on Ang-(1-7) infused mice, indicating that these animals already have reduced ROS levels [105]. This was further suggested by reduced levels of hydrogen peroxide and NAD(P)H oxidase subunit expression in Ang-(1-7)infused animals. In addition to reduced ROS activity, increased levels of eNOS were observed following treatment with Ang-(1-7), providing a further mechanism to support the findings of increased NO bioavailability [105]. Furthermore, Ang-(1-7) has recently been shown to modulate renal vascular resistance in Apo $E^{-/-}$ mice through inhibition of ROS mediated p38 MAPK activation [106].

In addition to enhancing vascular endothelial function, this increase in NO release has also been demonstrated to inhibit platelet aggregation, demonstrating an anti-thrombotic role for Ang-(1-7) [107]. Importantly, as well as mediating NO release from endothelial cells [104, 108] Ang-(1-7) has recently been shown to promote NO release from platelets, adding to its anti-thrombotic effects [107]. *In vivo* the anti-thrombotic effect of Ang-(1-7) was blocked by pharmacological Mas receptor inhibition and is absent in $Mas^{-/-}$ mice [107]. It was not established whether this was due to Ang-(1-7) actions in endothelial cells or platelets leading to NO release, although it is likely to be a combination of both [107]. While further work is required to fully dissect the mechanisms involved in the anti-thrombotic properties, these findings have identified the Ang-(1-7)/Mas axis as a potential therapeutic target for the treatment of thrombotic events. The recent development of an orally available form of Ang-(1-7) (Ang-(1-7)-CyD), in which Ang-(1-7) has been incorporated into a cyclodextrin (CyD), a cyclic oligosaccharide that enhances drug

stability, absorption across biological barriers and provides gastric protection, has greatly increased the potential of utilising Ang-(1-7) in a therapeutic setting [109]. This compound has been shown to be of particular use as an anti-thrombotic intervention as it exerts antithrombotic effects *in vivo,* associated with increased plasma levels of Ang-(1-7) [110].

Effects of Angiotensin-(1-9) in vascular remodeling

The role of Ang-(1-9) in the vasculature is relatively unexplored; however, as Ang-(1-9) can also exert biological effects, some of the previously described vasculoprotective effects of the counter-regulatory axis of the RAS may be attributed to production of both Ang-(1-9) and Ang-(1-7) (Figure 3). As in the heart, signalling via the AT_2R in the vasculature is poorly defined and further work is required to delineate a role in this setting (Figure 3). Inhibition of the RhoA/Rho-associated, coiled-coil containing protein kinase (ROCK) signaling pathway results in increased activity and expression of ACE2 in the aorta, and increased Ang-(1-9) plasma levels, reducing blood pressure and vascular remodeling [111]. These effects are coupled with reduced ACE activity, Ang II levels and increased expression of eNOS, identifying that the ROCK pathway may interact with the ACE2/Ang-(1-9) axis as a novel, protective interaction in the vasculature. Additionally, Ang-(1-9) has also been reported to indirectly contribute to improved vascular function by stimulating bradykinin release in endothelial cells and enhancing the effects of bradykinin by augmenting nitric oxide and arachidonic acid release [31]. While Ang II is largely accepted to be prothrombotic and Ang-(1-7) antithrombotic, evidence for Ang-(1-9) is inconclusive. A recent study by Kramkowski *et al* (2010) has indicated that Ang-(1-9) enhances electrically stimulated thrombosis and increases platelet aggregation in rats via the AT_1R [112]. However, it is worthwhile to point out that electrical stimulation was used to injure the vessel and initiate thrombosis which would not be the case clinically. Also, the pro-thrombotic effect of Ang-(1-9) was much lower than that of Ang II, as demonstrated in previous studies by the same group [113-114]. It was concluded that the actions of Ang-(1-9) were via metabolism to Ang II by an ACE independent aminopeptidase, a rare process, as opposed to direct actions of Ang-(1-9) [112, 115-116]. This may have implications for the therapeutic actions of Ang-(1-9) and requires further study.

Florez-Munoz *et al* (2012) recently provided the first evidence to suggest a direct beneficial effect for Ang-(1-9) in vascular function [65]. Ang-(1-9) infusion in the SHRSP improved aortic vasorelaxation and NO bioavailability via the AT_2R [65]. While the mechanisms involved are currently unknown it is possible that Ang-(1-9) may increase NO bioavailability by stimulating bradykinin release, as previously documented in cardiac endothelial cells [31], or by enhancing the activity of eNOS, as has been shown for Ang-(1-7) [117]. Additionally, Ang-(1-9) infusion and AT_2R stimulation resulted in an increase in aortic expression of NADPH oxidase 4 (NOX4) [65], which has been previously demonstrated to promote vasodilation via release of hydrogen peroxide [118]. However, this protective effect of NOX4 is vascular bed-specific [120] and therefore further investigation is required to fully assess the involvement of increased NOX4 in the aorta in response to Ang-(1-9) infusion [68].

Conclusion

In summary, clear evidence identifies the peptides of the counter-regulatory axis of the RAS as modulators of the pathological effects of Ang II in the heart and vasculature. The effects of Ang-(1-7), signaling via Mas are well established and there is clear evidence in support of its robust therapeutic potential in both cardiac and vascular remodeling. The identification of receptor-cross-talk pathways and potential interactions of Ang-(1-7) with other receptors may further highlight the broad therapeutic effect of Ang-(1-7), though further research is required to dissect the mechanisms involved. The generation of transgenic animals has greatly expanded our knowledge of Ang-(1-7) and enabled in depth investigation into its biological properties. Additionally, the production of orally available forms of Ang-(1-7) has increased the potential of targeting this axis clinically. Although developing therapeutic aspects of Ang-(1-7) is likely to be challenging due to the general rapid pharmacokinetics of angiotensin peptides *in vivo* other approaches are being developed. AVE0991 is the first non-peptide agonist of Mas which has been shown to have therapeutic effects in a range of disease models, including pulmonary, cardiac and renal injury and remodeling and in atherosclerosis [119-121]. Further development of these approaches is likely to provide key data to develop the Ang-(1-7)/ Mas axis as a clinical therapeutic target. More recently, Ang-(1-9) is emerging as another key peptide in this axis that exerts protective effects in the heart and vasculature, potentially via the AT_2R. While further work would be required to provide conclusive evidence in support of this interaction, Ang-(1-9) may represent an alternative therapeutic target within the counter-regulatory axis of the RAS in the setting of cardiac and vascular remodeling, with the potential of providing additive or synergistic effects to those mediated via Ang-(1-7).

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Figure 1

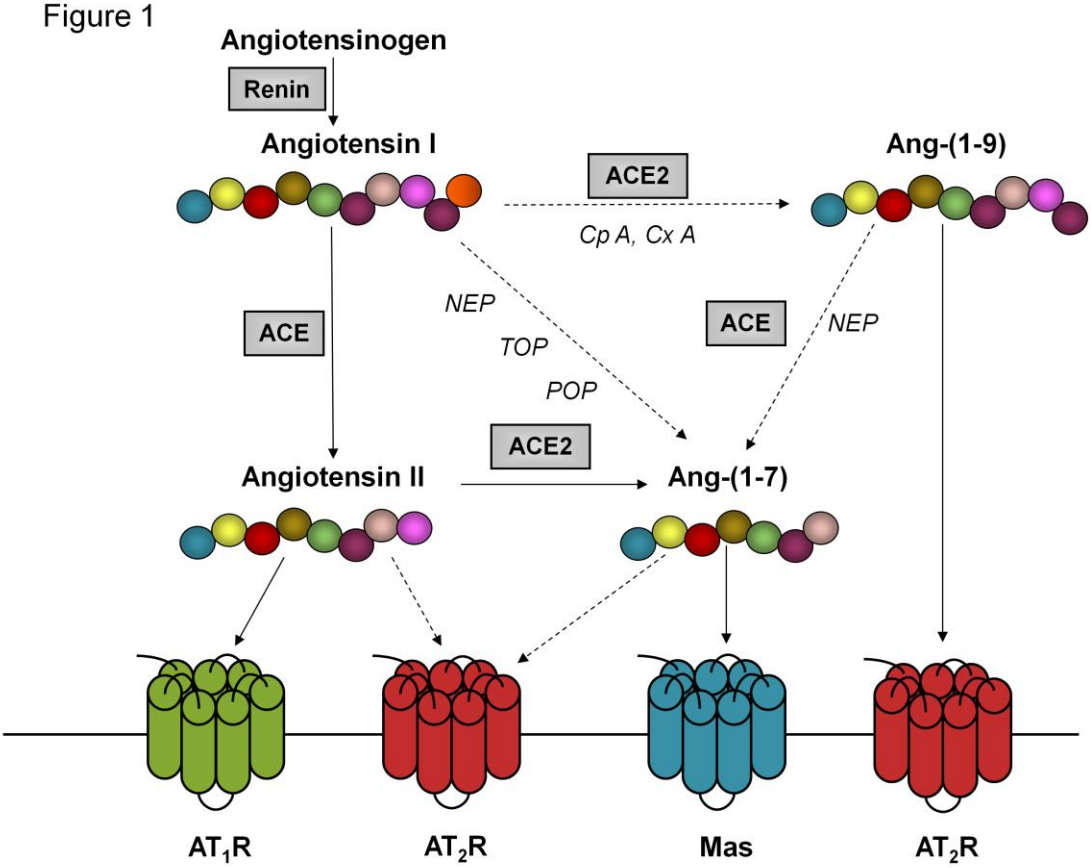

Figure 1: The renin angiotensin system. The renin-angiotensin system is a cascade of interconverted peptides that are generated mainly by the actions of ACE and ACE2. The traditional axis of the RAS is via ACE-mediated generation of Ang II which signals mainly via the AT_1R and also the AT_2R. The counter-regulatory axis of the RAS, is centered around the actions of ACE2 and production of Ang-(1-7) and Ang-(1-9) from Ang II and Ang I, respectively, and counteracts pathological effects of Ang II at the AT_1R. Ang-(1-7) exerts protective effects via Mas while Ang-(1-9) has recently been reported to act via the AT_2R. ACE: Angiotensin converting enzyme; Ang: Angiotensin; AT_1R: Angiotensin type 1 receptor; AT_2R: Angiotensin type 2 receptor; Cp A: Cathepsin A; Cx A: carboyxpeptidase A; NEP:Neutral endopeptidase; POP: Prolyl endopeptidase; TOP: Thimet oligopeptidase.

Figure 2

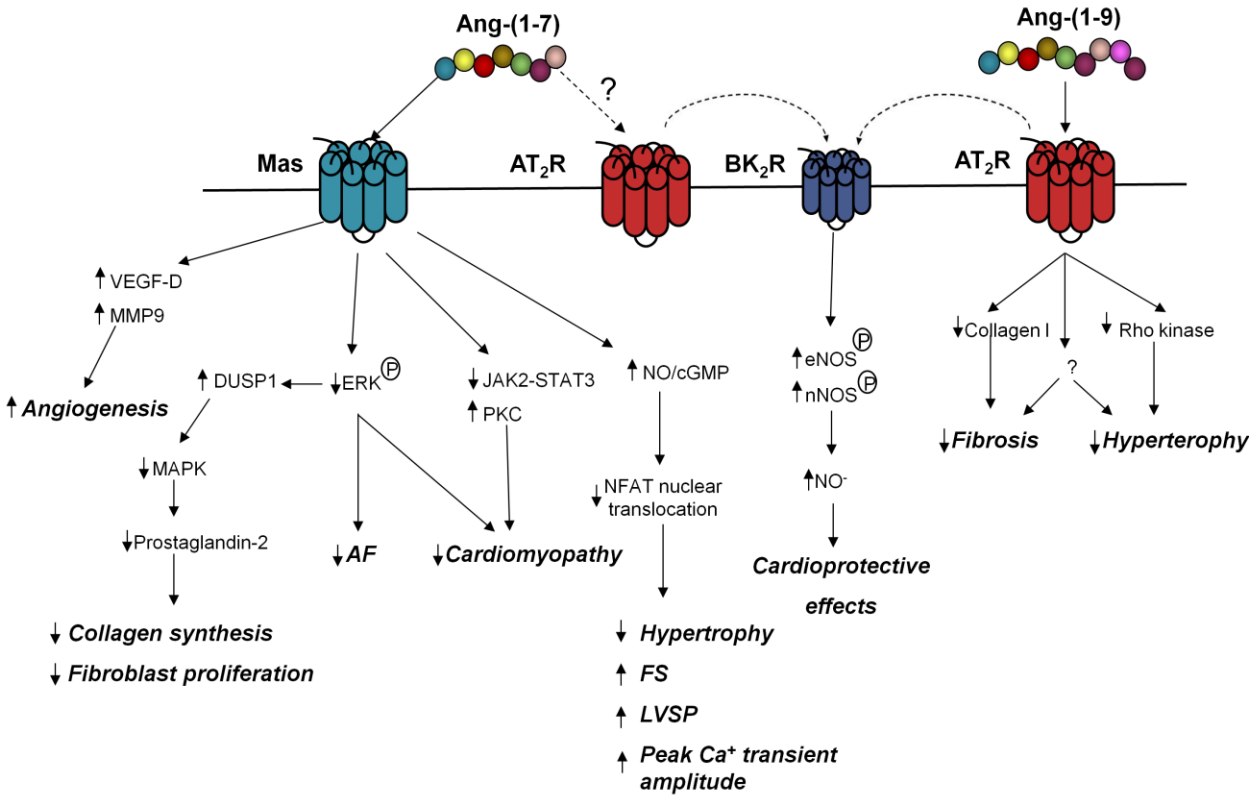

Figure 2: Angiotensin-(1-7) and angiotensin-(1-9) signaling in the heart. Ang-(1-7) acting at Mas regulates cardioprotective effects in the heart via NO release through cGMP, reducing NFAT translocation to the heart, thereby preventing cardiac hypertrophy, preventing reductions in cardiac FS, LVSP and Ca^{2+} transient amplitude in isolated cardiomyocytes. NO generation also occurs indirectly via Ang-(1-7) cross talk with the AT_2R and BK_2R which increases phosphorylation of eNOS and nNOS, also exerting cardioprotective effects. Ang-(1-7) signaling via Mas inhibits mitogen-activated JAK2-STAT3 and activates PKC, decreasing ERK activity which has been found to attenuate cardiomyopathy. Decreases in ERK activity has also been associated with reduced susceptibility to AF in animal models; as well as increasing DUSP1 activity resulting in a decrease in MAP-kinase activity reducing production of prostaglandin-2 which acts on fibroblasts, preventing proliferation and collagen synthesis. Via Mas, Ang-(1-7) has also been shown to increase VEGF-D and MMP9 levels, promoting cardiac angiogenesis. Ang-(1-9) signaling via the AT_2R reduces collagen synthesis thereby reducing cardiac fibrosis and decreases Rho kinase activity which attenuates cardiac hypertrophy, however the majority of Ang-(1-9) signaling pathways in the heart have yet to be characterized. AF: Atrial fibrillation; AT_2R: Angiotensin type 2 receptor; BK_2R: Bradykinin 2 receptor; cGMP: Cyclic guanosine monophosphate; DUSP1: Dual specificity phosphatase 1; FS: Fractional shortening; eNOS: endothelial nitric oxide synthase; LVSP: Left ventricular systolic pressure; MMP9: Matrix metalloproteinase 9; NFAT: Nuclear factor of activated T-cells; nNOS: Neuronal nitric oxide synthase; NO: Nitric oxide; PKC: Protein kinase C.

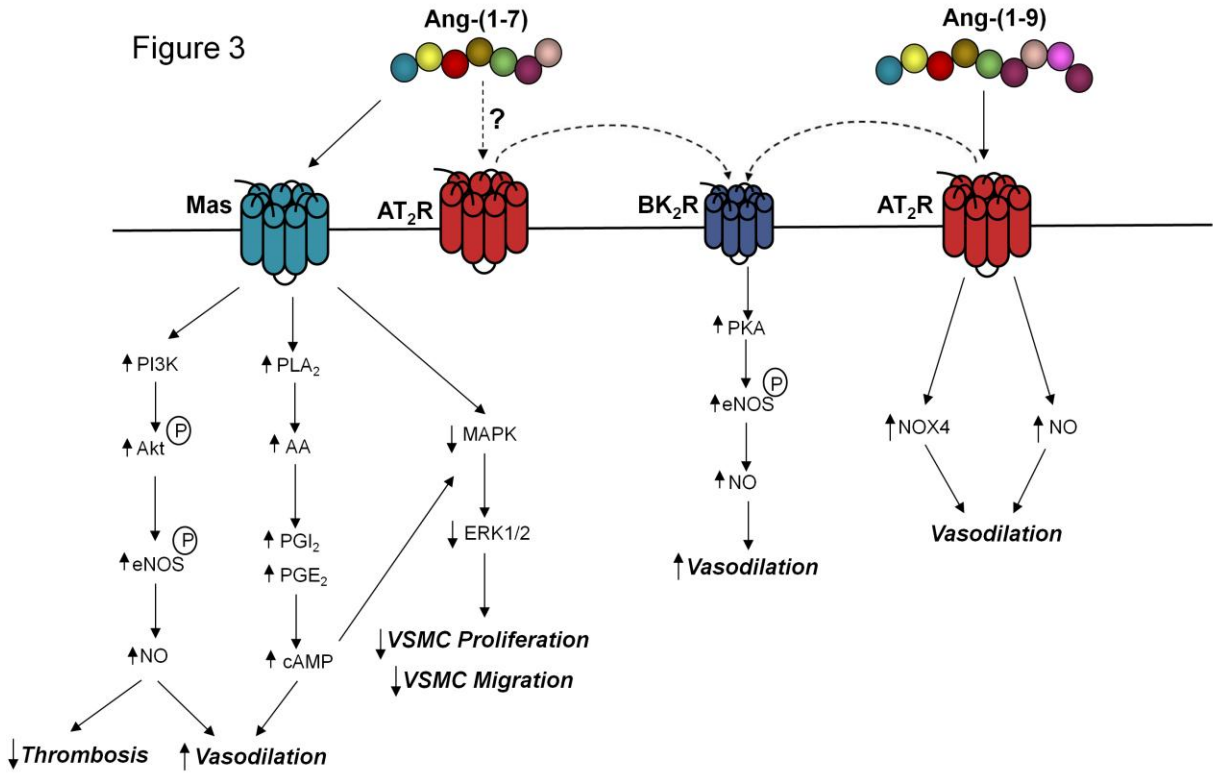

Figure 3: Angiotensin-(1-7) and angiotensin-(1-9) signaling in the vasculature. Ang-(1-7) via Mas increases NO release, thereby acting as a vasodilator and reducing thrombosis either 1) directly via activation of the PI3K/Akt signaling pathway resulting in increase phosphorylation of eNOS or 2) indirectly via cross talk with the BK_2R, which increases PKA mediated phosphorylation of eNOS- this pathway may involve also interact with the AT_2R. Ang-(1-7) via Mas also promotes vasodilation by stimulating PLA2 leading to increased release of AA, which in turn produces the prostanoids PGI2 and PGE2, both of which increase levels of cAMP. cAMP also reduces MAPK signaling and VSMC proliferation and migration. Ang-(1-7) via Mas also directly inhibits MAPK activity. Ang-(1-9) via the AT_2R promotes vasodilation through increased NO either directly or via crosstalk with the BK_2R. Ang-(1-9) mediated vasodilation is also associated with increased expression of NOX4. AA: Arachidonic acid; AT_2R: Angiotensin type 2 receptor; BK_2R: Bradykinin 2 receptor; cAMP: Cyclic adenosine monophosphate; eNOS: endothelial nitric oxide synthase; NO: Nitric oxide; NOX4: NADPH oxidase 4; PI3K: Phosphoinositide 3-kinase; PGI2: Prostacyclin: PGE2: Prostaglandin E2; PKA: Protein kinase A.