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Review Article

Angiotensin (1-7) and Alamandine: similarities and differences

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Abstract. A primary peptide of the renin angiotensin system (RAS), Angiotensin (Ang) II, is a vasoconstrictor and promotor of atherosclerosis. To counter this, the RAS also consists of peptides and receptors which increase nitric oxide release from the endothelium and decrease nicotinamide adenine dinucleotide phosphate oxidase-related superoxide production. Two peptides, Ang (1-7) and alamandine are vasodilators, by activating the nitric oxide pathway via different receptors in the endothelium. Thus, herein we focus on the similarities and differences between alamandine and Ang (1-7) and the counterbalancing hypothesis on Ang II during endothelial dysfunction and atherosclerosis.

Keywords: Renin angiotensin system; Angiotensin (1-7); Alamandine; Endothelial dysfunction

Chemical compounds mentioned in this article:

Alamandine (44192273); Angiotensin 1-7 (123805); Angiotensin II (172198); Angiotensin I (3081372)

Abbreviations:

Angiotensin A (Ang A); Angiotensin converting enzyme 1 (ACE 1); Angiotensin converting enzyme 2(ACE2); Angiotensin II type I receptor (AT_1); Angiotensin II type II receptor (AT_2); Cardiovascular disease (CVD); Chinse Hamster Ovary (CHO); Endothelial nitric oxide synthase (eNOS); Mas-related G couple protein receptor member D (MrgD); Nicotinamide adenine dinucleotide phosphate (NADPH); Nitric oxide (NO); Protein kinase A (PKA); Protein kinase C (PKC); Reactive oxygen species (ROS); Renin angiotensin system (RAS);Unilateral ureteral obstruction (UUO).

1.1 Introduction

Cardiovascular disease (CVD) accounts for 1 in 3 deaths worldwide [1] with increasing prevalence in the Western world. Despite the therapeutic advances for treating CVD [2], novel interventions are necessary to further reduce this burden. The renin angiotensin system (RAS) is a hormone system that regulates blood pressure, electrolyte balance and plays a crucial role in atherogenesis and thus CVD.

Atherosclerosis is a progressive process of arterial wall thickening and is characterised by the deposition of lipoproteins, cholesterol and white blood cells on the innermost layer of blood vessels [3]. Atherogenesis is complex, however 3 distinct stages have been identified. (1) Endothelial dysfunction, which occurs throughout all stages of atherosclerosis; (2) plaque formation, and, (3) thrombosis [4-6]. These stages occur at the luminal surface of blood vessels and are time-dependent [7]. Endothelial dysfunction is a pathophysiological shift towards a vasoconstrictive and pro-inflammatory state. This is believed to occur mainly due to a reduction in the bio-availability of nitric oxide, possible due to an altered activity of endothelial nitric oxide synthase (eNOS) [8] or an over-activation of the protein kinase C (PKC) signalling pathway [9] coupled with an overproduction of superoxide [10, 11]. Over-activation of RAS has been linked to endothelial dysfunction and may be involved in altering protein kinases, eNOS activity and superoxide production, ultimately regulating atherogenesis. Thus, drugs regulating RAS are a promising therapeutic modality against CVD.

The RAS includes a number of peptides and enzymes, including Ang II, angiotensin converting enzyme 1 (ACE1), ACE2, angiotensin A (Ang A, where the A is abbreviated for alanine), angiotensin (1-7) (Ang (1-7)), Mas receptor, Mas-related G coupled protein receptor member D (MrgD) and alamandine [12-14]. The RAS is present in a number of tissues including, blood vessels, myocardium, kidney and brain [15-18]. The pathway begins with angiotensinogen, which is formed in the liver and released into the circulation. It is proteolyzed by renin resulting in a the decapeptide, Ang I. Ang I binds to the endothelial cell layer of blood vessels, and ACE1 cleaves the C-terminal dipeptide (L-histidyl-L-leucine) of Ang I to form Ang II [19-21] (Figure 1).

Ang II and Ang A have vasoconstrictive properties, which act through the angiotensin II type I receptor (AT_1) [14, 22]. AT_1 is a 41 kDa transmembrane receptor highly expressed in the cardiovascular system and regulates aldosterone secretion and controls blood pressure [23]. Binding of Ang II to AT_1 receptor stimulates an array of pathophysiological actions including, vasoconstriction, which elevates blood pressure, endothelial dysfunction (inhibition of NO production) and increases nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation for superoxide production; leading to the development of cardiac fibrosis, inflammation associated with atherosclerosis and atherosclerotic plaques [24-28]. In addition, Ang II may act through the angiotensin II type II receptor (AT_2) resulting in vasodilatory effects. AT_2 is highly expressed in the developing fetus, however, its expression is low in the cardiovascular system but is increased in inflammation, hypertension and atherosclerosis and is vasoactive in human radial arteries [29]. Likewise, alamandine, is hydrolyzed by ACE2 from Ang A or decarboxylated from Ang (1-7), also exerts vasodilatory and anti-hypertensive actions, through the MrgD receptor [12, 30]. Ang (1-7) is also a vasodilator which acts via the Mas receptor, which is related to the MrgD receptor. Therefore, the localization of both the MrgD and the Mas receptor on endothelial cells suggests that both alamandine and Ang (1-7) may play important roles in blood vessel physiology [22, 31]. In addition, Ang (1-7) exerts an anti-angiogenic role by decreasing proangiogenic hormones such as vascular endothelial growth factor-A [12, 32]. However, there are no reports to demonstrate the effect of alamandine on angiogenesis. Furthermore, ingestion of alamandine/cyclodextrin decreases the accumulation of collagen I, III, and fibronectin in isoproterenol-treated rats, contributing to a decrease in cardiac fibrosis [12]. Interestingly, research performed in our laboratory showed a marked reduction in rat aortic wall elastic fibers after an atherogenic diet for 15 weeks [33], and thus it would be interesting to determine if alamandine can reduce this pathological effect. Therefore, Ang (1-7) and potentially alamandine acting via their specific receptors, may be able to decrease endothelial cell proliferation and migration, resulting in a decrease in endothelial cell permeability, and thus an attenuation of atherogenesis.

Both the Mas and the MrgD receptors are proto-oncogenes. The Mas proto-oncogene was first identified in 1986 where it was noted to facilitate tumorigenesis, and similarly for the overexpression of the MrgD receptor in murine fibroblast cell lines [34, 35]. Thus, if alamandine and Ang (1-7) are to be used as a treatment for CVD, these peptides could affect oncogenesis. Thus, it is important to determine a possible carcinogenic/oncogenic effect in animal models using alamandine and Ang (1-7) in a combined CVD/cancer model.

Ang (1-7) counterbalances the effects of Ang II exerted on human cerebral smooth muscle cells [25]. These effects reduce Ang II-induced apoptosis and proliferation [25]. Indeed, *in vitro* studies show that administration of Ang (1-7) to rat aorta prevents Ang II-stimulated reactive oxygen species (ROS) production [24] and increases NO release resulting in increased vasodilation [36], and reduces endothelial oxidative stress. We are unaware of studies that demonstrate alamandine counterbalancing Ang II effects on vascular smooth muscle cells and on endothelial cells. It is possible that alamandine may mimic the effects of Ang (1-7), due to the close homology of their amino-acid sequence (Figure 1 and 2). Indeed, alamandine stimulates NO release potentially by eNOS in endothelial cells to induce vasodilation through the MrgD receptor [12]. Thus, combined Ang (1-7)/alamandine would be viable pathways to reduce Ang II-associated endothelial dysfunction (i.e. low NO release from endothelium), Ang II-associated vascular proliferation, and Ang II-associated inflammation during atherosclerosis.

1.2 Biosynthetic pathway for of Ang (1-7) and Alamandine

The RAS exists in the circulatory and tissue system and circulatory-Ang II is synthesized when ACE cleaves the C terminal dipeptide of the renin-catalyzed-angiotensin I [37]. An intracellular form of renin, as well as angiotensin I and both forms of ACE expressed in tissues have been associated with increased intracellular Ang II production [38]. Therefore, the formation Ang (1-7) and alamandine, can also be biosynthesized at a circulatory and tissue level. As revised by Zhang et al (2015), ACE2 catalyzes angiotensin (1-9) from angiotensin I, so that angiotensin (1-9) can be cleaved by ACE into Ang (1-7).

Moreover, Ang II can be directly converted into Ang (1-7) via ACE2 [39]. Also, carboxylase can convert Ang (1-7) to alamandine. Similarly to Ang (1-7), alamandine can be synthesized at the circulatory and tissue system (Figure 1).

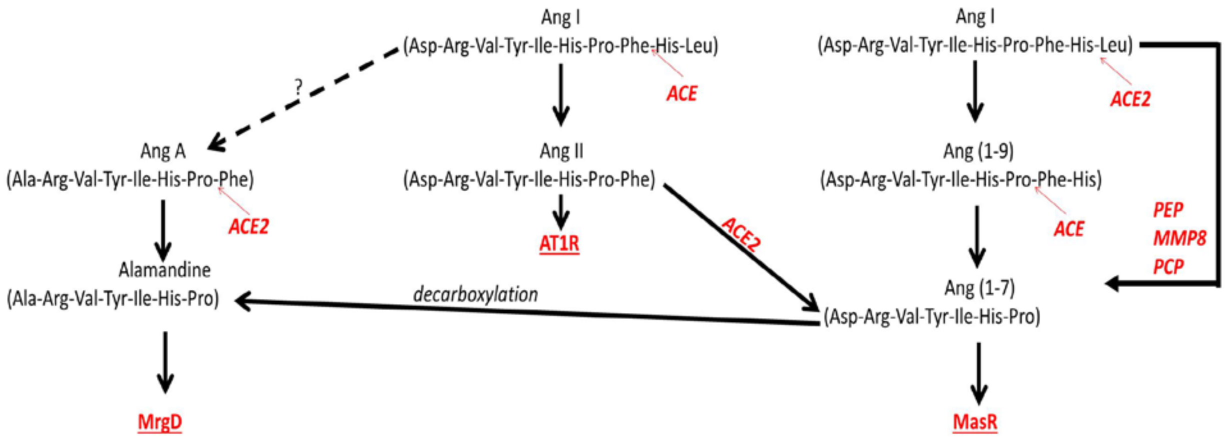

Figure 1: The pathway for both alamandine and Ang (1-7). Whether Ang A is formed from Ang I remains unknown. However, Ang A has been demonstrated through chromatographic purification and structural analysis that it is mostly likely generated enzymatically from Ang II via decarboxylase [14]. Ang I can be cleaved into Ang (1-7) by prolyl endopeptidase (PEP) [40], matrix metalloproteinase-8 (MMP8) [41] and prolyl carboxypeptidase (PCP) [42].

1.3 Factors involved in vascular dysfunction, leading to atherosclerosis

The widely studied vasodilator produced by the endothelial layer of blood vessels is the free radical gas, NO [43]. NO contributes to normal vascular homeostasis and reduced NO bioavailability results in endothelial dysfunction [44, 45]. CVD risk factors, such as type 2 diabetes, hypertension, and hypercholesteremia exacerbate the reduction of NO bioavailability thus promoting atherosclerosis. Since atherosclerosis is the primary cause of CVD, the pharmacological drive to reverse endothelial dysfunction, by improving NO release, should improve vascular health and reduce the burden of CVD. In addition, ROS released by cells, including endothelial cells, is a byproduct of oxygen in normal homeostasis. During stress however, ROS is produced in excessive levels leading to cell damage (cellular oxidative stress). In fact, overproduction of Ang II leads to dramatic increase in ROS production by endothelial cells, through the NADPH oxidase enzyme inducing cellular oxidative stress, leading to endothelial dysfunction, endothelial apoptosis and promotion of thrombosis formation [46-48]. Hence, cellular oxidative stress and reduction in NO, contribute to endothelial dysfunction [49-51]. Furthermore, RAS peptides and enzymes are increased in cardiovascular disease in tissues, such as, blood vessels [22], kidney [52], cardiomyocytes [53], skeletal muscle cells [54] and the colon [55], hence treatment modalities are targeted to reduce the systemic over-activation of the RAS.

1.4 Trophic effects of RAS peptides on endothelial cell function and vascular tone regulation

Vascular endothelial cells release trophic factors such as NO, neutral endopeptidase, prostaglandin I2 and endothelin-1 that regulate vascular tone. An imbalance of these vasoactive factors may lead to an imbalance of vascular tone and induce proliferation of VSMCs, leading to hypertension and atherosclerotic plaque accumulation. Although physiological levels of Ang II, upon stimulation of the AT_1 and AT_2 receptors on endothelial cells signal vascular tone and proliferation, pathophysiological levels of Ang II disrupts this homeostasis and induces excess VSMC proliferation in rodents, rabbits and humans [56-58]. In mice and rat, two weeks of Ang II infusion via mini-osmotic pumps induces hypertension, cardiac and VSMC hypertrophy, and endothelial dysfunction [59]. Indeed, in humans, an imbalance between Ang (1-7)/Ang II ratio has been shown in systemic sclerosis-induced endothelial dysfunction [60]. Furthermore, Ang(1-7) infusion inhibits VSMC proliferation in balloon-injured carotid arteries of rats [61]. Although further evidence is required to fully elucidate the mechanistic pathway elicited by Ang (1-7), most *in vitro* studies conducted on human or rodent cultured aortic endothelial cells show that after Ang (1-7) and its associated receptor signalling on the endothelial cell surface, an array of enzymes are phosphorylated, such as phosphoinositide 3-kinase/AKT, protein kinase A, phospholipase A2 phosphorylation leading to increased production of eNOS-induced NO and cyclic

AMP production [36, 62]. Ang (1-7) has also been shown to cause a reduction of mitogen-activated protein kinase (MAPK) phosphorylation concomitant with a reduction of extracellular-signal-regulated kinase (ERK1/2) phosphorylation within endothelial cells [62, 63], which is likely to be a major anti-proliferation pathway induced by Ang (1-7).

A critical role for Ang (1-7)/Ang II has been established in endothelial dysfunction and cellular proliferation. Considering research from this laboratory is clearly providing a vasoactive role for both alamandine and Ang A [22], a trophic or anti-trophic effect on endothelial cells has not as yet been demonstrated. Therefore, future experiments similar to studies conducted for Ang (1-7) will provide the essential information necessary to compare these two heptapeptides.

1.5 Ang (1-7) and Mas receptor counterbalance the effects of Ang II and AT_1 receptor

The Mas receptor is a G-coupled protein receptor which is expressed in brain, kidneys, testes, heart and blood vessels of both humans and mice [36, 64-67]. It is conceivable that Ang (1-7) mediated through the Mas receptor [68], may have beneficial effects in the treatment for CVD. Indeed, Ang (1-7) at 80nM has been shown to protect against hypoxia and cardiomyocyte apoptosis by preventing ROS-induced mitochondrial dysfunction in rat cell lines [69]. This suggests that Ang (1-7) may be protective in specific tissues and that it may act on mitochondria. Furthermore, a number of RAS blockers have been developed in order to reduce the effects of Ang II; such blockers (renin inhibitors, ACE inhibitors and Ang II receptor blockers) all have anti-hypertensive effects [15]. Interestingly, inhibition of Ang II via ACE1 blockers increases Ang (1-7) by 5–25-fold in blood [70] and Ang (1-7) may also be involved in the anti-depressant effect of ACE1 treatment in hypertensive rats [71]. This suggests that Ang (1-7) alone has an important role in blood pressure regulation via inducing vasodilation protecting endothelial function and hence, cardiovascular related diseases. Since Ang (1-7) is formed by ACE1, increasing its levels could be a viable alternative to inhibition of ACE1 in order to achieve desired plasma Ang (1-7) concentration. Likewise, alamandine has anti-hypertensive properties, enhances vasodilation and reverses endothelial dysfunction in spontaneous hypertensive rats and homocysteine-induced endothelial dysfunction rabbits [12, 72]. Hence, as Ang (1-7) can reverse the negative effects of Ang II on endothelial dysfunction, superoxide production, as well as lowering Ang II-induced atherosclerotic lesions and plaque formation, further studies with alamandine could show similar findings, and together these heptapeptides could reduce the progression of CVD.

However, controversial results on Ang (1-7)-beneficial physiological effects have been reported. In Mas knockout mice induced with unilateral ureteral obstruction (UUO) kidneys, the administration of Ang (1-7) worsened renal lesions compared to untreated Mas knockout UUO mice [73], suggesting that Ang (1-7) administration can be harmful in individuals with obstructed kidneys. However, one must take into account that Ang (1-7) has only been shown to exert beneficial physiological effects, such as reducing atherosclerotic plaque and inducing vasodilation, in the presence of the Mas receptor. Additionally, it has been shown that mice with UUO already develops severe renal inflammation with significant upregulation of inflammatory chemokines and cytokines such as MCP-1 and IL-6 respectively [74], suggesting that Ang (1-7) may not be a beneficial treatment at severe stages of disease, and that it may potentially exacerbate the development of disease instead. However, more studies report that chronic treatment of Ang (1-7) is able to reduce fat accumulation and inflammation in animal models of liver disease, suggesting Ang (1-7) as a strong anti-inflammatory agent [75]. Thus the model chosen plays a role in the controversial reports of any experiments related to Ang (1-7) as a treatment.

Of interest, Ang (1-7) and Ang II both stimulated ERK1/2 phosphorylation and enhanced mesangial cell proliferation [76], further conflicting most reports that demonstrate the opposing effects of Ang (1-7) compared to Ang II [77, 78]. However, when co-administered to rat renal mesangial cells, Ang (1-7) inhibited the stimulatory effects induced by Ang II, which could be blocked by a Mas receptor blocker, A779, yet anti-AT_1 or anti-AT_2 receptor blockers had no effect [76]. Hence, although interacting with each other [79], Ang (1-7) specifically acts on the Mas receptor and Ang II specifically acts on the AT_1 receptor. In human umbilical vein endothelial cells (HUVEC), Ang (1-7) at 1μM had no effect on endothelial cell apoptosis, however when HUVEC are treated with Ang II, Ang (1-7) suppresses apoptosis [80]. This indicates not only the crucial role on endothelial cell survival [62], but also that Ang (1-7) either interacts with the AT_1 receptor by dislodging Ang II binding or interacts with the Mas receptor. Similar findings were observed in aortic vascular cells of ACE2-knockout mice [81]. Furthermore, in Ang II induced endothelial dysfunction, blocking of the AT_1 receptor augments the production of NO and improves vasodilation, which are crucial factors in the prevention of hypertension and atherosclerosis [82, 83]. Since, blocking of the AT_1 receptor is associated with an increase of Ang (1-7), the addition of Ang (1-7) may counterbalance the effects of Ang II induced endothelial dysfunction and may aid in the prevention of atherosclerosis. To this effect, *ex vivo* treatment of murine aortae with Ang II impairs vasodilation which is reversed by Ang (1-7) [31]; these effects are diminished following treatment with the Mas receptor antagonist, A779. These findings, suggest that endothelial dysfunction induced by Ang II (reduced NO and vasodilation), could be counterbalanced by Ang (1-7) acting through the Mas receptor. Moreover, the studies suggest that the counterbalancing effects caused by Ang (1-7) through the Mas receptor are a compensatory system that is important to balance the over-activity of Ang II through the AT_1 receptor. However, the cellular mechanism underlying this theory is not yet clear, although some studies suggest a specific non-competitive inhibition of Ang (1-7) on the AT_1 receptor [84, 85]. Others suggest that the AT_1 receptor and Mas receptor form a hetero-oligomer in order for their effects to be counterbalanced [76, 86]. Further research to establish these initial results are necessary.

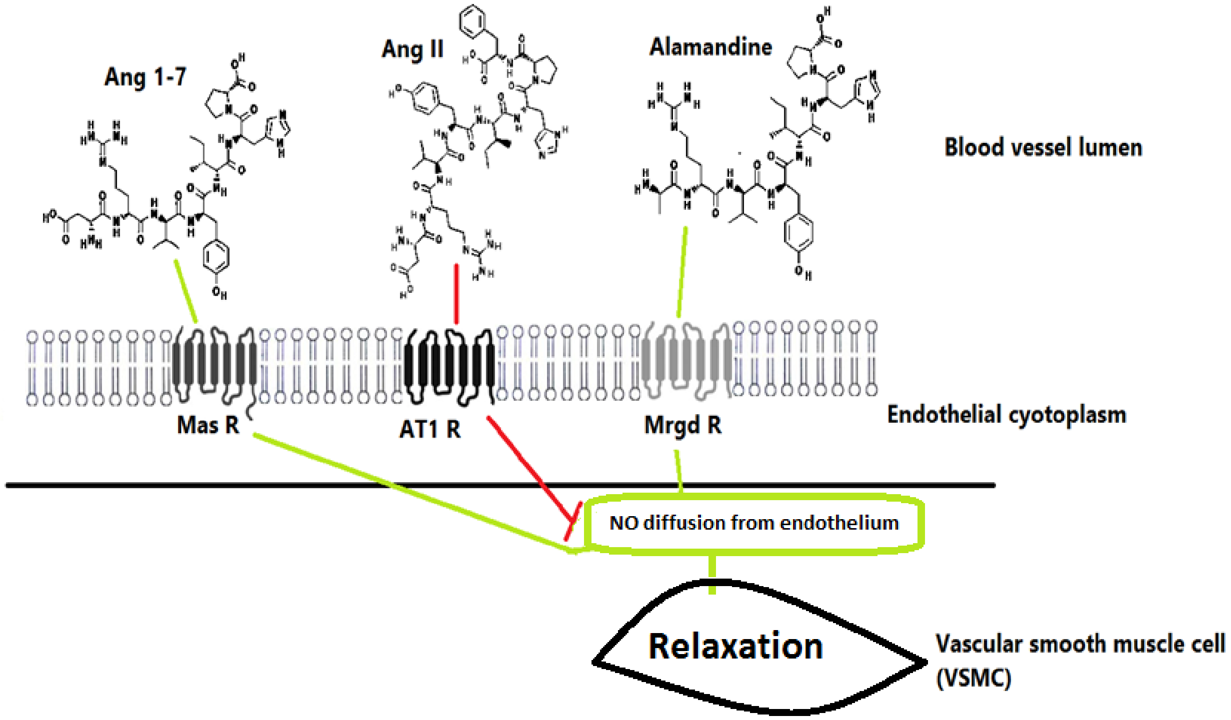

Figure 2: The chemical structure of Ang II, Ang (1-7) and alamandine peptides as ligands and their corresponding receptors. Both Ang (1-7) and alamandine have similar amino acid sequence and they both function as ligands for the proto-oncogene receptors, Mas and MrgD, respectively. Upon stimulation of these receptors there is upregulation of NO from the endothelium of blood vessels. The release of NO diffuses into the vascular smooth muscle cell (VSMC) layer (as NO is a soluble gas) and induces relaxation. However, Ang II counters these effects upon stimulation of the AT_1 receptor. (NO, nitric oxide; VSMC, vascular smooth muscle cell; Red line, blocking effect; Green line, activation).

1.6 Alamandine and the MrgD receptor: similarities to Ang (1-7)

Alamandine, a heptapeptide, is closely related to the vasodilator Ang (1-7), with only one amino acid difference, whereby the first amino acid of Ang (1-7) is aspartate, and alanine is for alamandine. Therefore, alamandine can be synthesized from Ang (1-7) by decarboxylation of aspartate into alanine. As these peptides have similar amino acid sequence and structure (Figure 1 and 2), their effects are highly likely to be similar. Through chromatographic approach, it was demonstrated that a peptidase exists in the medulla of sheep brain which converts Ang (1-7) into Ang (1-4) [87]. Although the peptidase was not identified, it was suggested that metalloendopeptidase may have been responsible. More recently, it was noted that the hydrolysis of alamandine may also occur via metalloendopeptidase for Ang (1-7) in kidney tissue [88].

Interestingly, Chinese Hamster Ovary (CHO) cells transfected with Mas receptor and incubated with Ang (1-7) resulted in elevated NO levels and eNOS activity, which was inhibited by the Mas receptor blocker , A779. In addition, wortmannin, a specific phosphatidylinositol 3 (PI3) kinase inhibitor reduced NO levels and eNOS activity in the absence of A779 [36]. Similar effects were also noted in human aortic endothelial cells (HAEC), which constitutively express the Mas receptor [36], as well as in rat hearts [89]. In addition, Ang (1-7) through the Mas receptor activates the protein kinase B pathway (PKB) in pancreatic islet endothelial cells [62], increasing eNOS activity and NO production. However, in mesangial cells Ang (1-7) activates Mas receptor via a different protein kinase pathway, PKA, and correlates with increased cyclic AMP [90].

To further understand the similar roles amongst Ang (1-7) and alamandine, we recently demonstrated that *ex vivo* treatment of alamandine to isolated rabbit aortas reversed homocysteine-induced endothelial dysfunction by activating the PKA pathway for vasorelaxation [72]. Although we demonstrated the presence of MrgD receptor using immunohistochemistry in another study [22], others have shown that CHO cells transfected with MrgD receptor and cultured with alamandine, high levels of NO is produced but not in normal CHO cells, suggesting that alamandine binds to MrgD receptor to induce NO production via increasing eNOS activity [12]. This specific finding establishes that alamandine binds to MrgD receptor and that the outcome is enhanced NO production through eNOS, the enzyme that produces NO within endothelial cells. Whilst these studies were conducted in non-diseased cells [12], we previously demonstrated that alamandine was not able to prevent endothelial dysfunction in atherogenic-diet fed rabbits, however alamandine is likely to exert more acetylcholine-mediated vasodilation in thoracic and carotid artery compared to acetylcholine-exerted vasodilation alone in these atherogenic vessels [22]. These studies clearly suggest that the role of alamandine may exert beneficial vasodilatory effects on the initial steps of atherosclerosis and may not play a role in advanced stages of atherosclerosis. Possibly, the function and expressions of MrgD receptor on the endothelial cell layer may not be sufficient or efficient when the vessel is completely dysfunctional and, therefore, alamandine has no beneficial effects in such conditions. Hence, early intervention needs to be considered when treating atherosclerosis with alamandine.

Moreover, in organ bath experiments, individual incubations of alamandine and Ang (1-7) were not able to reduce Ang II mediated vasoconstriction [22]. Conversely, Ang (1-7) has been shown to reverse Ang II-mediated endothelial dysfunction in mouse aorta [31]. Although the results are conflicting, differing experimental conditions exist. In our study [22], the vessels were incubated with the RAS peptides and then a dose-response curve to Ang II was constructed, whereas in [31], the vessels were initially incubated with Ang II in organ cultures for 24 hours and then Ang (1-7) was incubated, where 30 minutes after this point a dose-response curve to acetylcholine was constructed [31]. These differences suggest that further studies are required to elucidate the roles of alamandine and Ang (1-7) during diseased states such as atherosclerosis.

1.7 Conclusion

Studies from our and other laboratories show that Ang (1-7) and alamandine are able to promote beneficial effects on the cardiovascular system, such as, counterbalancing Ang II-associated endothelial dysfunction and therefore, may reduce the development of Ang II associated atherosclerosis.

Table 1: A summary of the novel renin angiotensin system (RAS) components and their function and mechanisms on endothelial cells

RAS peptides	**Reference**	**Induce or**	**Other cardiovascular**	**Mechanism**

and receptors		prevent endothelial dysfunction?	harmful or protective effects?	
Ang II	[46-48]	Induce	Involved in hypertension, smooth muscle cell proliferation	Through NADPH oxidase, reduction in NO
Ang A	[14, 22]	Remains unknown	Vasoconstrictor	Possibly through the AT_1 receptor
Ang 1-7	[22, 31]	Prevent	Anti-proliferative effects, vasodilation	Counterbalance Ang II actions by increasing eNOS activity, and thus NO production
Alamandine	[12, 22, 72]	Prevent	Anti-hypertensive effects, vasodilation	May potentially counterbalance Ang-II actions
AT1 receptor	[82, 83]	Upon agonistic stimulation; induce	Upon agonistic stimulation, vasoconstriction	Superoxide production, increase in NADPH oxidase expression
Mas receptor	[36]	Prevent	Upon agonistic stimulation, vasodilation	Increase in eNOS activity, NO production, and reduction in superoxide production
MrgD receptor	[12, 72]	Prevent	Upon agonistic stimulation, vasodilation	Increase in eNOS activity, NO production via PKA pathway

Ang, Angiotensin; AT_1, Angiotensin II type I receptor; eNOS, endothelial nitric oxide synthase; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; PKA, protein kinase A.

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