The Angiotensin-Converting Enzyme 2/Angiotensin-(1–7)/Mas receptor axis: A potential target for treating thrombotic diseases

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Summary
Despite many therapeutic advances leading to increasingly effective drug treatments, thrombotic events (such as ischaemic stroke, pulmonary embolism, deep venous thrombosis and acute myocardial infarction) still represent a major worldwide cause of morbidity and mortality. Remarkable effort has been made to identify new drug targets. There is growing evidence indicating that the recently described counter-regulator axis of the renin-angiotensin system (RAS), composed of Angiotensin-Converting Enzyme 2 (ACE2), Angiotensin-(1–7) and the Mas receptor, has protective effects against thrombosis. In addition, it could be considered as a promising target for treating or preventing this disease. In this narrative review, we focused on the recent findings of the role of the ACE2/Angiotensin-(1–7)/Mas axis on the haemostatic process and its therapeutic potential.

Keywords
Angiotensin-(1–7), Angiotensin Converting Enzyme 2, Receptor Mas, thrombosis, platelet

Introduction
Thrombotic events (such as ischaemic stroke, pulmonary embolism, deep venous thrombosis, mesenteric ischaemia and acute coronary syndrome) are the major complications of some chronic cardiovascular diseases (CVD), such as hypertension, atherosclerosis and diabetes mellitus (1, 2). Despite various therapeutic advances that have led to increasingly effective drug treatments, thrombogenic events still represent a major worldwide cause of morbidity and mortality (3, 4).

Haemostatic disorders are common in several vascular diseases (3). In normal circumstances, the intact endothelium prevents platelet adhesion and activation through several mechanisms, including endothelial cell production of prostacyclin (PGI2) and nitric oxide (NO) (5). Endothelial disruption and deep intimal injury caused by plaque rupture lead to the exposure of components, such as collagen and von Willebrand factor (vWF) (6). Subsequently, platelets directly adhere to collagen or vWF via glycoprotein Ib-V-IX complex. Local platelet activation stimulates additional platelet recruitment by releasing potent mediators, such as adenosine diphosphate, serotonin, and thromboxane A2. The activation of the coagulation cascade occurs concomitantly, leading to fibrin strand formation, which strengthens the platelet plug (7). Under pathological conditions, the hyperactive haemostasis process may result in an exacerbation of the thrombus formation and consequently clinical thrombotic syndromes (3, 5).

The renin-angiotensin system (RAS) is a major hormonal regulator of blood pressure and hydro-electrolyte balance (8), and it also participates in haemostasis (9). It has been reported that an increased activity of some RAS components is associated with a higher risk of acute thrombosis by destabilizing atherosclerotic plaques (10), promoting endothelial dysfunction and enhancing platelet activity and coagulation (10, 11).

RAS supports a series of enzymatic reactions that culminate in the generation of Angiotensin (Ang) II by the angiotensin converting enzyme (ACE). Ang II is the main effector of RAS, acting primarily through the AT1 receptor and promoting adverse cardiovascular phenotypes (12). Currently, this classical concept of a linear renin-angiotensin axis has been expanded, including novel players, such as ACE2 (13–15) (an ACE homologue enzyme) and the Mas receptor (16). ACE2 catalyses the conversion of Ang II to Ang-(1–7) (13, 17, 18). Ang-(1–7) has been re-

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ported to counter-regulate Ang II actions (19–21), thereby promoting many beneficial cardiovascular outcomes through interactions with the G-protein coupled receptor (GPCR) Mas (16, 17, 19). In contrast with the detrimental actions promoted by the ACE/Ang II/AT1 axis, it has been reported that the activation of ACE2/Ang-(1–7)/Mas axis might mediate antiplatelet activity (9, 22–24). Therefore, this novel system has been suggested as a potential target for the treatment of thrombogenic diseases. The aim of this narrative review is to highlight the recent findings addressing the role of the ACE2/Angiotensin-(1–7)/Mas axis on the haemostatic process and its therapeutic potential. In order to attain this goal, we performed serial literature searches for English and non-English articles via PubMed (http://www.ncbi.nlm.nih.gov/ pubmed/) (1950–present). The terms searched with Boolean logic included “angiotensin”, “angiotensin-converting enzyme 2”, “thrombosis”, “Mas receptor”, “platelet”, “lymphocyte” and “reactive oxygen species”.

Renin angiotensin system (RAS)

The RAS is a peptide hormone system, which plays a central role regulating both cardiovascular and haemostatic systems (9, 25). Acting through an endocrine, paracrine or autocrine manner, this system modulates the function of numerous cells, such as cardiomyocytes, fibroblasts, platelets and inflammatory and vascular cells by exerting influential control in the maintenance of homeostasis (11, 14, 26, 27). The generation of the RAS peptides is initiated by the conversion of the angiotensinogen to the inactive decapetide, Ang I, through the enzymatic action of renin (26). Subsequently, Ang I is cleaved by the angiotensin-converting enzyme (ACE) forming the octapeptide, Ang II (28), the main effector of the RAS (26). The actions of Ang II are mediated through two GPCRs, AT1 and AT2 (29, 30); however, most of the cardiovascular effects of Ang II are mediated by the AT1 receptor (31, 32). In pathological conditions, AT1 receptor activation has been shown to induce deleterious effects, such as vasoconstriction (26), oxidative stress (33), platelet aggregation (34) and exacerbated thrombus formation (11). Ang II may also bind to the AT2 receptor, generally resulting in opposing effects when compared to the actions mediated by the AT1 receptor activation (35, 36). However, a comprehensive understanding of the role of AT2 receptor has not been fully elucidated.

The relevance of this axis is highlighted by the success obtained by therapeutic strategies against CVD based on the pharmacological inhibition of ACE and blockade of AT1 (37–39). However, in these last two decades, this simple concept of a linear RAS action has increased its complexity (17). The “traditional” RAS concept has been expanded by findings that demonstrate the existence of previously unknown signal transduction pathways, alternative metabolic cascades and the discovery of additional components (17, 26). It has been proposed that, in addition to the ACE/Ang II/AT1 receptor axis, the RAS has a counter regulatory axis composed by ACE2, Ang-(1–7) and the Mas receptor (21).

Ang-(1–7) is a biologically active component of the RAS, which binds to the Mas GPCR (16) inducing many beneficial actions, including vasodilation (16, 40), reduction of oxidative stress (41, 42) and antithrombotic effects (22). In this manner, Ang-(1–7)/Mas counter-regulates the effects of Ang II/AT1 (17, 21, 43). Ang-(1–7) is mainly produced through the action of ACE2 (13). This enzyme cleaves the C-terminal phenylalanine from Ang II, leading to Ang-(1–7) formation (Fig. 1). Ang-(1–7) may also be formed directly from Ang II by prolylcarboxypeptidase (PCP) and prolylendopeptidase (PEP) (44) or from Ang I by neutral endopeptidase (NEP) and PEP (20). However, it has been accepted that the major enzyme responsible for Ang-(1–7) formation is ACE2 (17). This enzyme has approximately 400-fold higher affinity to Ang II than to Ang I (13), indicating that Ang II is the major substrate for Ang-(1–7) synthesis, therefore ACE2 directly contributes to the balance between the two antagonistic RAS peptides (17, 21, 26, 43).

Importantly, a chronic and sustained imbalance between these opposing RAS axes may contribute to the development of CVD (17) and haemostatic disturbances. Indeed, an increased activity of ACE/Ang II/AT1 has been associated with higher risk of thrombosis (11), whereas a decrease in ACE2 activity was associated with a prothrombotic condition in spontaneously hypertensive rats (23). Moreover, Ang-(1–7) mediated activation of the Mas receptor has demonstrated robust antithrombotic activity (22).

ACE/ANG-II/AT1 Axis in haemostasis

The ACE/Ang II/AT1 axis, which has been shown to play a well-established role in the progression of hypertension, seems also to be involved in thrombogenic processes (11, 45). Indeed, ACE inhibitors reduce by 20% the incidence of thrombotic occlusive events, such as acute myocardial infarction and stroke (45).

Clinical studies have shown that the inhibition of RAS components not only improves hypertension, but also protects against atherothrombotic diseases. In fact, ACE inhibitor (ACEi) and AT1 receptor blocker (ARB) therapy improve the prothrombotic state in hypertensive patients (11, 46).

In particular, ACEi treatment enhances fibrinolysis, through favouring t-PA release over PAI-1. Treatments with quinapril, perindopril or enalapril enhanced the release of bradikinin-induced t-PA in human circulation (47) and blocked the expression of PAI-1 through inhibition of Ang II production (48–51). However, this issue is still controversial. Treatment with ARB (such as losartan, telmisartan or irbesartan) has been shown to reduce PAI-1 (46, 50, 52–54), while other authors did not observe any difference in PAI-1 or t-PA levels during AT1 antagonist therapy (47). Moreover, when PAI-1 levels decrease due to losartan treatment, its effect is not persistent for more than six weeks (55). Thus, the role of AT1 receptor antagonists in fibrinolysis is still uncertain.

Endothelial dysfunction is another thrombogenic feature that can be improved by ACEi, but not ARB therapy (56–59). Quinapril or perindopril treatment (ACEi drugs), decreased the levels of endothelial dysfunction markers, such as endothelin-1 or plasma...
vWF antigen, respectively (56, 57), possibly activating bradykinin-dependent mechanisms (60). Different experimental data also showed that long-lasting treatment with perindopril had protective effects in vascular endothelium (60, 61).

Inhibition of ACE by perindopril is also involved in the reduction of platelet aggregation (1, 56, 62–64). On the other hand, clinical studies investigating ARB (such as telmisartan) did not attenuate platelet responsiveness (56). However, some controversial data demonstrated an inhibition of platelet adhesion and aggregation elicited by losartan through competitive antagonism of thromboxane A2 receptor (65). Other ARBs (such as EXP3174 and valsartan) also reduced platelet aggregation (66, 67).

Thus, classical RAS antagonists have been shown to reduce prothrombotic conditions, beyond their role in normalising elevated blood pressure.

ACE2/ANG-(1–7)/MAS axis in haemostasis

As discussed above, the ACEi and ARBs have protective effects against thrombosis. Nevertheless, the mechanism of action of these drugs goes beyond the inhibition of Ang II. Chronic treatment with ACEi and ARBs directly increases the concentration of Ang-(1–7) by five- to 50-fold (68, 69). Indeed, previous findings demonstrated that A-779, a Mas receptor antagonist, attenuated the inhibition of thrombus formation obtained by both captopril and losartan administration in the abdominal vena cava of renovascular hypertensive rats (2-kidney 1-clip) (24). These results strongly suggest that the intrinsic antithrombotic effect of ACEi and ARBs are mediated, at least in part, by an increase in Ang-(1–7) levels. In addition, this study demonstrates that the activation of the ACE2/Ang-(1–7)/Mas axis could be a potential therapeutic target against thrombosis.

The initial study demonstrating the direct antithrombotic effect of Ang-(1–7) was performed by Kucharewicz et al. (70). They observed that the intravenous infusion of Ang-(1–7) reduced the weight of the thrombus induced by the ligature of the abdominal vena cava in renovascular hypertensive rats (24, 70). However, Ang-(1–7) did not have any observed effect in normotensive rats (24). Additionally, the antithrombotic effect of Ang-(1–7) was reversed by A-779 in a dose-dependent manner, but not by PD123319 (an AT2 receptor antagonist) (24). We recently demonstrated that the antithrombotic effect of Ang-(1–7) was absent in Mas receptor gene-deleted (Mas-/-) mice, revealing that this effect of Ang-(1–7) strictly depends on the Mas receptor (22). Moreover, we found that the bleeding time of Mas-/- mice was reduced compared to wild-type mice, suggesting an important role of the Mas receptor, counteracting the haemostatic processes (22).

The mechanisms regarding the antithrombotic effect of Ang-(1–7) are not completely understood. Previous reports su-
port an active role of NO and prostacyclin (PGI₂), both of which are important antiplatelet agents in the haemostatic process (1, 5, 6). Concomitant pre-treatments with NO synthase inhibitor (L-NAME) and PGI₂ synthesis inhibitor (indomethacin) completely reversed the inhibition of thrombus formation by Ang-(1–7) intravenous infusion (24). The involvement of NO and PGI₂ is in accordance with a number of studies, indicating the direct mediation of these pathways in Ang-(1–7) vascular activity. Acting through the Mas receptor, Ang-(1–7) stimulates PGI₂ production (16, 71) and NO release from endothelial cell via activation of PI3K/Akt/eNOS pathway (72).

In addition to this activity on endothelial cells, evidence suggests that Ang-(1–7) may also directly modulate platelet activity. Recently, we detected the presence of the Mas receptor on platelets (22). More importantly, we demonstrated that the Mas receptor activation by Ang-(1–7) increased the production of NO in platelets (22) (Fig. 2). Furthermore, it has been reported that incubation of platelet preparations with increasing concentrations of Ang-(1–7) reduced their adhesion to fibrillar collagen (70). Further studies have observed that Ang-(1–7) alone did not affect platelet aggregation stimulated by the thromboxane A2 mimetic, U46619, but potentiated the anti-aggregation effects of the NO donor, sodium nitroprusside (73). Taken together, these data suggest that the antiplatelet effect of Ang-(1–7) is dependent on the activation of multiple pathways. Generally, it appears that the anti-thrombotic effect of Ang-(1–7) is due to its interaction with the Mas receptor in both platelets and endothelial cells. Mas receptor activation generates NO in platelets (22) and both NO and PGI₂ in endothelial cells (71, 72). Importantly, these mediators might inhibit platelet activation (5, 6), thus complicating the role of each cell type in the Mas receptor-activated antithrombotic pathway.

Intriguingly, dose-dependent response curves for the anti-thrombotic effect of Ang-(1–7) displayed a bell-shaped response. Several studies reported that the inhibitory effect of Ang-(1–7) on thrombus formation was abolished when higher doses of Ang-(1–7) were administered (22, 24, 74). In addition, the same phenomenon was observed in platelet activation (73). The mechanism involved in this biphasic effect of Ang-(1–7) has not been addressed; however, it does not appear to be mediated by the desensitisation of the Mas receptor, since prolonged treatment (eight weeks) with Ang-(1–7) produced an even more robust antithrombotic effect compared to the acute treatment (74). Importantly, this limitation needs to be taken into account for future therapeutic strategies targeting Ang-(1–7)/Mas.

ACE2, the major enzyme responsible for the generation of Ang-(1–7) (13), is also involved in the pathophysiological formation of a thrombus. A decrease in ACE2 activity, but not in ACE2 protein expression, was associated with increased thrombus formation in spontaneously hypertensive rats (23). Supporting that finding, the pharmacological activation of ACE2 was associ-

Figure 2: Schematic illustration showing the mechanism for the anti-thrombotic effect of ACE2/Ang-(1–7)/Mas axis. HPBβCD-Ang-(1–7): inclusion compound with Ang-(1–7) in hydroxypropyl β-cyclodextrin, angiotensin II; Ang-(1–7): angiotensin-(1–7); ACE2: angiotensin-converting enzyme 2; XNT: ACE2 activator [93]; DX-600: ACE2 inhibitor; Mas: Ang-(1–7) receptor; A-779: D-ala-angiotensin-(1–7), a Mas antagonist; PI3K: phosphatidylinositol 3-kinases; Akt: protein kinase B; NO: nitric oxide; PGI2: prostacyclin; IP: prostacyclin receptor; cGMP: cyclic guanosine monophosphate; cAMP: cyclic adenosine monophosphate; (-): inhibition or inhibitory pathway; (+): activation or stimulatory pathway.
ated with a marked reduction of thrombus formation in spontaneously hypertensive rats (23). In addition, pre-treatment with DX-600 (Fig. 2), an ACE2 inhibitor, increased the thrombus formation in both hypertensive and normotensive rats (23). Moreover, ACE2 also modulates platelet activity, since its activation decreased platelet attachment to injured microvessels (23). It is generally assumed that the beneficial effect of ACE2 in thrombosis is primarily due to an increased production of Ang-(1–7) with concomitant degradation of Ang II. However, these mechanisms still need to be further addressed (see the proposed mechanism for potential antithrombotic effect of ACE2/Ang-(1–7)/Mas axis in the Fig. 2).

Recent studies pointed out that RAS plays a key role on haemostatic processes within the microvasculature (75, 76). For instance, chronic infusion of Ang II enhanced thrombus development in mouse microvasculature (75). Interestingly, this effect of Ang II was mediated by circulating CD4+ and CD8+ lymphocytes and linked to ROS generation from NADPH oxidase (76). Since Ang-(1–7) often counter-regulates the effects evoked by Ang II (17, 20, 21), these novel findings suggested that ACE2/Ang-(1–7)/Mas might potentially modulate the haemostatic process in the microvasculature through mechanisms involving the adaptive immune system. In particular, direct prothrombotic activities of Angiotensin II on T-lymphocytes require further confirmative pathophysiological studies. Although type 1 Angiotensin receptor-mediated pathway on T lymphocytes has been shown to potentially protect the kidney from the hypertensive damage (77), the expression and potential bioactivity of Mas receptor on these cells remain unknown. In addition, the pathophysiological role of T lymphocyte-generated oxidative stress on the expression of ACE2 and Mas receptor on either endothelial cells or platelets might represent an intriguing investigation field for researchers not only in CVD (42), but also in essential hypertension.

Therapeutic strategies

Many studies have demonstrated the beneficial effects of Ang-(1–7), suggesting that this peptide could be a potential tool for treating CVD (78, 79). However, the therapeutic use of this peptide is limited due to its unfavorable pharmacokinetic properties. Ang-(1–7) is rapidly cleaved by peptidases, possessing a half-life of about 10–15 seconds (80). In addition, Ang-(1–7) may not be orally administered due to its degradation while passing through the gastrointestinal tract. To solve these impediments, new strategies have been implemented to develop a more feasible application of Ang-(1–7) in practice.

Recently, our group developed a formulation based on the Ang-(1–7) included into hydroxypropyl-β-cyclodextrin [HPβCD/Ang-(1–7)] (81). Cyclodextrins (CyDs) are pharmaceutical tools that can be used in formulation to enhance the stability of the drug, the absorption across biological barriers and to afford gastric protection (82–84). They are cyclic oligosaccharides, which consist of six to eight glucoseanose units forming a truncated cone shaped molecule with a contrasting polar exterior and an apolar cavity (82, 83). Its amphiphilic property allows the formation of supramolecular complexes that are stabilised by non-covalent interactions with a variety of hydrophobic molecules (83, 85). CyDs are neither hydrolysed nor absorbed in the stomach or the small intestine. However, the colon microflora breaks these compounds into smaller saccharides, delivering the guest molecule, Ang-(1–7) in this case, into the large intestine where it will be absorbed (81). Therefore, the formulation HPβCD/Ang-(1–7) was developed aiming at the oral administration of Ang-(1–7). Recently, we have shown that acute and chronic oral administration of HPβCD/Ang-(1–7) promotes a strong antithrombotic effect reducing the thrombus weight induced by ligation in spontaneously hypertensive rats (74). In addition, we observed that the antithrombotic effect of this formulation was strictly mediated by Mas receptor, since it was abolised in Mas−/− mice, contrasting with a full response in wild type mice (74). Additionally, these effects were associated with a significant increase in Ang-(1–7) plasma levels (74).

Alternative strategies to administer Ang-(1–7) have also been proposed, including a liposomal delivery system (86, 87) and a cyclic Ang-(1–7) derivative (88), which presented an increase in stability and preserved activity. However, the effects of these compounds against thrombus formation have not been evaluated yet.

Synthetic Mas receptor agonists have also been developed. AVE-0991 (89) and CGEN-8565 (90) are unique compounds that compete with Ang-(1–7) for the same binding site in Mas-transfected cells (90, 91). The drugs markedly and selectively induce vasodilation via Mas receptor-dependent pathways in mice (90, 92). Since these compounds mimic Ang-(1–7)-mediated actions, they are potential antithrombotic agents. However, the effect of these compounds needs to be studied in more detail.

Another pharmacological strategy targeting the ACE2/Ang-(1–7)/Mas axis was recently proposed by Hernández-Prada et al. (93). Based on the crystal structure of ACE2 and using a virtual screening strategy, they identified molecules that may interact with this enzyme, resulting in enhanced activity (90). The antithrombotic effect of the ACE2 activator, XNT (Fig. 2), was tested in both hypertensive and normotensive rats, which resulted in a diminished thrombus formation in the abdominal vena cava (23). Moreover, this compound decreased platelet adhesion to FeCl3-injured microvessels from the dorsal skin of nude mice (23).

Future perspectives

The above discussion supports the concept that the vasoprotective activity of the ACE2/Ang-(1–7)/Mas receptor axis can produce beneficial effects against thrombosis pathophysiology. This system has been also shown as a pharmacological target in animal experiments. However, the molecular mechanisms underlying the direct inhibition of thrombus formation by ACE2/Ang-(1–7)/Mas axis has not been clarified yet. Many intriguing hints of investigation might be considered:
It appears that both endothelium and platelets might actively contribute to the antithrombotic effect evoked by Ang-(1–7)/Mas pathway. However, the selective contribution of each cell type has to be elucidated. The use of bone marrow chimeras in Mas gene-deleted mice would be interesting to address this issue. Moreover, the role of other cell types, such as T-lymphocytes, has to be better evaluated.

Mas receptor activation has been shown to promote NO release from platelets (22). Therefore, it would be interesting to determine if Mas activation would change the intracellular calcium influx or the released contents from platelet granules.

Ang II has been shown to enhance thrombus development in the microvasculature mediated by CD4+ and CD8+ lymphocytes and ROS generation (92). Since Ang-(1–7) might induce an opposite effect to Ang II (20, 21), it would be interesting to determine if ACE2/Ang-(1–7)/Mas receptor axis may directly modulate the haemostatic process in the microvasculature involving the adaptive immune system.

The activity of ACE2 is decreased in the thrombus from hypertensive rats, but not its protein expression (23). The evaluation of the expression and activity of ACE2 or ACE2 in activated platelets would be of particular importance to clarify the potential connections between hypertension and thrombosis.

The recently discovered compounds selectively targeting ACE2/Mas receptor activation have been shown to potently inhibit the thrombus formation. Might these novel medications be more efficient than ACEI (already available on the market)? Superiority trials have to be performed.

Is the benefit of ACE2/Mas intervention organ specific? The influence of ACE2 activators and Mas agonists on different organs (such as the heart, kidney and vessels) needs to be taken in account when evaluating its antithrombotic effect.

The selective pharmacological activation of ACE2 promotes remarkable benefits due to an increased Ang-(1–7) production with concomitant degradation of Ang II. However, the molecular pathways activated by ACE2 inducers and their potential effects on Ang II and Ang-(1–7) levels remain to be clarified.

**Conclusion**

Growing evidence indicates that the novel ACE2/Ang-(1–7)/Mas receptor axis is a crucial component of a more complex RAS, with both autocrine and paracrine regulations. The ACE2/Ang-(1–7)/Mas receptor system has been shown to induce protective effects against thrombosis, and may be considered as a potential target for treating or preventing this disease. However, ongoing studies are underway to further understand the mechanisms by which this axis participates in the haemostatic processes.

**Conflicts of interest**

None declared.

**References**


