

Angiotensin-converting enzyme 2, angiotensin-(1–7) and Mas: new players of the renin-angiotensin system

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Abstract

Angiotensin (Ang)-(1–7) is now recognized as a biologically active component of the renin-angiotensin system (RAS). Ang-(1–7) appears to play a central role in the RAS because it exerts a vast array of actions, many of them opposite to those attributed to the main effector peptide of the RAS, Ang II. The discovery of the Ang-converting enzyme (ACE) homolog ACE2 brought to light an important metabolic pathway responsible for Ang-(1–7) synthesis. This enzyme can form Ang-(1–7) from Ang II or less efficiently through hydrolysis of Ang I to Ang-(1–9) with subsequent Ang-(1–7) formation by ACE. In addition, it is now well established that the G protein-coupled receptor Mas is a functional binding site for Ang-(1–7). Thus, the axis formed by ACE2/Ang-(1–7)/Mas appears to represent an endogenous counterregulatory pathway within the RAS, the actions of which are in opposition to the vasoconstrictor/proliferative arm of the RAS consisting of ACE, Ang II, and AT₁ receptor. In this brief review, we will discuss recent findings related to the biological role of the ACE2/Ang-(1–7)/Mas arm in the cardiovascular and renal systems, as well as in metabolism. In addition, we will highlight the potential interactions of Ang-(1–7) and Mas with AT₁ and AT₂ receptors.

Key Words

- ▶ Angiotensin II
- ▶ ACE2
- ▶ Mas
- ▶ Cardiovascular functions
- ▶ Metabolism

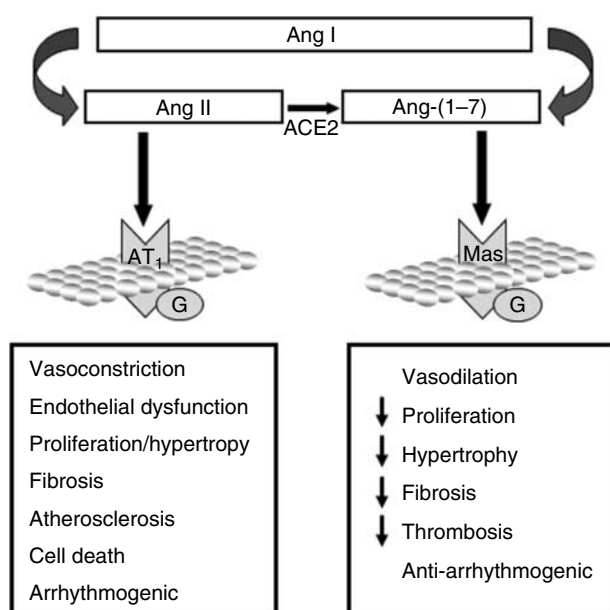
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Introduction

Santos *et al.* (1988) described the formation of the heptapeptide angiotensin (Ang)-(1–7) from Ang I by an Ang-converting enzyme (ACE)-independent pathway. In the same year, Schiavone *et al.* (1988) published the first report of a biological action of this heptapeptide *in vitro*, release of vasopressin from hypothalamus-neurohypophyseal explants. One year later, Chappell *et al.* (1989) discovered the peptide in the rat brain and Campagnole-Santos *et al.* (1989) described the first *in vivo* action of Ang-(1–7) using microinjection in the nucleus tractus solitarius (nTS) of anesthetized rats. These seminal studies

and many others that followed led to the recognition of Ang-(1–7) as a biologically active peptide of the renin-angiotensin system (RAS; Carey & Siragy 2003, Santos *et al.* 2005, Ferrario 2006, Bader 2010).

The identification of the ACE homolog, ACE2, as a key Ang-(1–7)-forming enzyme unravels the existence of a distinct enzymatic pathway for the production of this peptide (Donoghue *et al.* 2000, Tipnis *et al.* 2000). This monocarboxypeptidase can remove the amino acid leucine from the C-terminus of Ang I to form the biologically active peptide Ang-(1–9) (Donoghue *et al.*

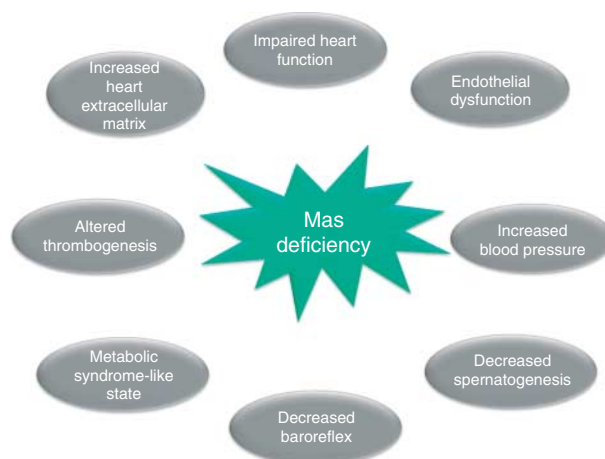
**Figure 2**

Opposing cardiovascular effects of the two major peptides of the renin-angiotensin system, Ang II and Ang-(1-7). The intersection between these two arms of the system is the angiotensin-converting enzyme 2 (ACE2), since this enzyme can cleave the vasoconstrictor/proliferative peptide Ang II to form the vasodilator/antiproliferative fragment Ang-(1-7). AT₁, Ang II type 1 receptor; Mas, Ang-(1-7) receptor. Ang, angiotensin.

local generation of Ang-(1-7) have been demonstrated in hearts from dogs (Santos *et al.* 1990), rats (Neves *et al.* 1995, Mahmood *et al.* 2002, Averill *et al.* 2003, Mendes *et al.* 2005) and human (Zisman *et al.* 2003, Campbell *et al.* 2004). Immunoreactive Ang-(1-7) was found in aortic root, coronary sinus and right atrium of dogs at basal conditions and its levels were markedly reduced following treatment with the ACE inhibitor CGS-14831 (Santos *et al.* 1990). Ang I is extensively metabolized during a single pass through the coronary bed leading to the generation of Ang II, Ang III, Ang IV and Ang-(1-7) in isolated hearts from normal (Neves *et al.* 1995, Mahmood *et al.* 2002) and diabetic rats (Mahmood *et al.* 2002). In addition, immunohistochemical staining revealed that Ang-(1-7) is expressed in rat cardiac myocytes (Averill *et al.* 2003) and sinoatrial node cells (Ferreira *et al.* 2012). Ang-(1-7) is also formed in human hearts and ACE inhibitors markedly decrease Ang-(1-7) generation, suggesting a substrate preference for Ang II (Zisman *et al.* 2003) although contrasting evidence has also been presented (Campbell *et al.* 2004), which may be due to the different tissue preparations (homogenates or coronary bed) used in both studies. Of note, the Ang-(1-7) receptor Mas, mRNA and

protein (Metzger *et al.* 1995, Santos *et al.* 2006, Ferreira *et al.* 2012), is localized in cardiac tissues as well as ACE2, the main Ang-(1-7)-forming enzyme utilizing Ang II as substrate (Harmer *et al.* 2002, Garabelli *et al.* 2008, Ferreira *et al.* 2012).

Recent studies report that ACE2 is an important regulator of cardiac pathophysiology (Crackower *et al.* 2002, Yamamoto *et al.* 2006). However, it should be stressed that the role of ACE2 in heart function and structure might depend on the species (Gurley *et al.* 2006). Interestingly, ACE2 expression has been reported to be increased in failing human heart ventricle (Zisman *et al.* 2003, Goulter *et al.* 2004, Burrell *et al.* 2005). Nevertheless, there are contrasting findings in rat hearts. While an increase of both ACE and ACE2 was found by Burrell *et al.* (2005) in hearts from Sprague-Dawley rats after myocardial infarction, Ishiyama *et al.* (2004) observed an increase in ACE2 expression only after AT₁ blockade in Lewis normotensive rats. These divergent results further suggest that ACE2 effects are strain dependent. ACE2 gene transfection using lentiviral vectors significantly attenuated cardiac damage in SHR (Diez-Freire *et al.* 2006) and in Ang II-infused Sprague-Dawley rats (Huentelman *et al.* 2005). Also, the stage of the disease apparently influences the expression of ACE2. At the early phase of myocardial infarction, ACE2 activity in plasma and left ventricles is increased in rats while the plasma and left ventricular ACE2 activities and mRNA levels are lower than in controls at 8 weeks postinfarction (Ocaranza *et al.* 2006). Similar findings were observed regarding the cardiac expression of Mas, i.e. it changes depending on the nature

**Figure 3**

Phenotypic changes observed in mice with gene-targeted deletion of Mas.

and duration of the physiological and pathological stimuli (Dias-Peixoto *et al.* 2012).

The actions of Ang-(1-7) in coronary vessels include biochemical and functional alterations leading to vasodilatation either directly in artery rings or indirectly through bradykinin (BK) potentiation or by opposing Ang II actions (Santos *et al.* 2000). In isolated canine coronary artery rings precontracted with the thromboxane A₂ analog, U46619, Ang-(1-7) elicited a dose-dependent vasorelaxation, which was completely blocked by the nonselective Ang II antagonist [Sar¹,Thr⁸]-Ang II, but not by the selective AT₁ or AT₂ antagonists, CV11974 and PD 123319 respectively (Brosnihan *et al.* 1996). This heptapeptide induced a concentration-dependent dilator response in porcine coronary artery rings, which were markedly attenuated by nitric oxide (NO) inhibition (Porsti *et al.* 1994). However, Gorelik *et al.* (1998) observed a vasodilator effect of Ang-(1-7) only in BK-stimulated pig coronary artery rings. Furthermore, Ang-(1-7) elicited an increase in the vasodilator effect of BK in isolated perfused rat hearts. This effect was dependent on Mas and NO and prostaglandin release (Almeida *et al.* 2000). Ang-(1-7) also evoked vasodilation in isolated perfused mouse hearts involving interaction of Mas with AT₁- and AT₂-related mechanisms (Castro *et al.* 2005). Together, these data suggest that Ang-(1-7) is a vasorelaxant peptide in the coronary bed and that this effect involves coupling to Mas and release of NO and prostaglandins. Nevertheless, because Neves *et al.* (1997) found that, at high concentrations (>25 nM), Ang-(1-7) induces a concentration-dependent decrease in coronary flow in isolated perfused rat hearts, it remains to be demonstrated whether Ang-(1-7) directly causes vasodilation in the coronary bed. This effect was not accompanied by consistent changes in contraction force and heart rate. A similar finding was observed in isolated hamster hearts (Kumagai *et al.* 1990). Thus, low concentrations of Ang-(1-7) should be tested to clarify this important issue.

Recent reports have indicated that heart tissue is also an important target for ACE2/Ang-(1-7)/Mas actions. It has been demonstrated that Ang-(1-7) decreases the incidence and duration of ischemia-reperfusion arrhythmias in isolated perfused rat hearts (Ferreira *et al.* 2001) apparently involving activation of the sodium pump (De Mello 2004). These effects were abolished by ouabain (De Mello 2004). Additionally, Ang-(1-7) decreased total (Na⁺, K⁺, Mg²⁺)-ATPase activity in sheep atrium (Lopez Ordieres *et al.* 1998). Also, the antiarrhythmogenic effect of Ang-(1-7) was blocked by the Ang-(1-7) antagonist A-779 and by the cyclooxygenase (COX) inhibitor

indomethacin (Ferreira *et al.* 2001). Importantly, it has been reported that all components of the ACE2/Ang-(1-7)/Mas axis are expressed in sinoatrial cells of rats, thereby providing morphological support to the antiarrhythmogenic effect of Ang-(1-7) (Ferreira *et al.* 2012). This peptide also improved postischemic contractile function in isolated heart preparations by a mechanism involving Mas and the release of BK and prostaglandins (Ferreira *et al.* 2002). However, at concentrations ~10 000-fold higher, Neves *et al.* (1997) found that Ang-(1-7) facilitated reperfusion arrhythmias in isolated perfused rat hearts. In keeping with this latter data, transgenic mice overexpressing ACE2 in the heart presented sudden death due to cardiac arrhythmias (Donoghue *et al.* 2003). These observations suggest that only very high local concentrations of Ang-(1-7) exert deleterious effects in the heart possibly through activation of NADPH oxidase (Oudot *et al.* 2005) or release of norepinephrine (Gironacci *et al.* 1994). In fact, transgenic rats presenting a local increase of Ang-(1-7) of up to 20-fold in the heart did not show any sign of arrhythmias (Ferreira *et al.* 2010).

Ang-(1-7) produced a significant increase in cardiac output and stroke volume in Wistar rats, partially attenuated by A-779 (Sampaio *et al.* 2003). These effects were also observed in transgenic rats that express a fusion protein capable of releasing Ang-(1-7) into circulating blood, increasing plasma concentration of this peptide 2.5-fold (Botelho-Santos *et al.* 2007). In addition, these animals showed a slight, but significant, increase in daily and nocturnal dP/dt, more resistance to isoproterenol-induced cardiac hypertrophy, reduced duration of reperfusion arrhythmias, and improved postischemic function in isolated perfused hearts (Santos *et al.* 2004), further supporting a beneficial role for Ang-(1-7) in cardiac function at physiological concentrations. According to Loot *et al.* (2002) chronic infusion (8 weeks) of Ang-(1-7) improved coronary perfusion and preserved cardiac function in an experimental rat model of heart failure induced by ligation of the left coronary artery. The vascular endothelial dysfunction observed in aortic rings from rats with myocardial infarction was also reversed by chronic infusion of Ang-(1-7) (Loot *et al.* 2002). In addition, Ang-(1-7) immunoreactivity was significantly increased in the tissue surrounding the infarct area of rat hearts with myocardial infarction (Averill *et al.* 2003).

Wiemer *et al.* (2002) published the first study demonstrating that the compound AVE 0991 is a nonpeptide and orally active Ang-(1-7) receptor agonist that mimics the Ang-(1-7) effects in bovine endothelial cells. Pinheiro *et al.* (2004) and Lemos *et al.* (2005) reported

that this compound acts as a Mas agonist in the kidney and isolated aortic rings respectively. We have observed that AVE 0991 preserved cardiac function and attenuated the development of hypertrophy and fibrosis in hearts from rats chronically treated with isoproterenol (Ferreira *et al.* 2007b). This nonpeptide Ang-(1-7) analog also significantly improved the cardiac function in hearts subjected to myocardial infarction and preserved the myocardium after ischemia (Ferreira *et al.* 2007a). Furthermore, long-term treatment with AVE 0991 prevented the end-organ damage in hearts from spontaneously hypertensive rats treated with L-NAME (Benter *et al.* 2006).

Recently, it has been shown that the inclusion of Ang-(1-7) into the cavity formed by the oligosaccharide hydroxypropyl β -cyclodextrin (HP β CD) could protect the peptide during the passage through the gastrointestinal tract. Taking advantage of this formulation, Marques *et al.* (2011, 2012) found that chronic oral administration of HP β CD/Ang-(1-7) significantly attenuated the impairment of heart function and cardiac remodeling induced by isoproterenol treatment and myocardial infarction in rats. Furthermore, CGEN-856S, a Mas agonist, promoted antiarrhythmic effects and produced a small dose-dependent decrease in arterial pressure in conscious SHR (Savergnini *et al.* 2010). Interestingly, activation of intrinsic ACE2 using the compound XNT improved the cardiac function of spontaneously hypertensive rats (SHR; Hernandez Prada *et al.* 2008) and of diabetic rats (Murca *et al.* 2012a,b).

One of the most important beneficial effects of Ang-(1-7) is its ability to regulate the expression of extracellular matrix proteins and cardiac remodeling. Iwata *et al.* (2005) reported that Ang-(1-7) binds to isolated adult rat cardiac fibroblasts, which play a critical role in cardiac remodeling. Treatment of these cells with Ang-(1-7) inhibited Ang II-induced increases in collagen synthesis. Ang-(1-7) also attenuated either fetal bovine serum- or endothelin 1-stimulated ^3H -leucine incorporation into isolated neonatal rat cardiac myocytes through a mechanism involving inhibition of serum-stimulated ERK1/2 MAP kinase activity and activation of Mas (Tallant *et al.* 2005). Chronic administration of this peptide significantly attenuated left ventricular hypertrophy and fibrosis in pressure-overloaded rats (Wang *et al.* 2005) and fibrosis in Ang II-infused and deoxycorticosterone acetate (DOCA)-salt rats (Grobe *et al.* 2006, 2007). Importantly, deletion of Mas produced impairment of cardiac function associated with a significant increase in collagen type I, III and fibronectin content in the heart (Santos *et al.* 2006, Gava *et al.* 2012). On the other hand, ACE2 activation

using XNT decreased cardiac fibrosis induced by high blood pressure through a mechanism involving reduction of ERK1/2 expression (Hernandez Prada *et al.* 2008, Ferreira *et al.* 2011). Altogether, these findings indicate that the ACE2/Ang-(1-7)/Mas axis is a functional cardioprotective arm of the RAS (Fig. 2).

The signal transduction pathways following activation of Mas in the heart are not fully characterized, but probably involve release of prostacyclin and/or NO release (Almeida *et al.* 2000, Ferreira *et al.* 2001, Castro *et al.* 2005) since Ang-(1-7) stimulated NO production and activated endothelial NO synthase (eNOS) and Akt in cardiomyocytes (Dias-Peixoto *et al.* 2008). Of note, the antihypertrophic effects of Ang-(1-7) on Ang II-treated cardiomyocytes were prevented by the blockade of the NO/cGMP pathway (Gomes *et al.* 2010). Moreover, amplification of the actions of BK (Gorelik *et al.* 1998, Almeida *et al.* 2000) and decrease of Ang II levels in the heart (Mendes *et al.* 2005, Nadu *et al.* 2008) may also be possible mechanisms involved in the beneficial cardiac effects of Ang-(1-7).

Vascular actions of the ACE2/Ang-(1-7)/Mas axis

Blood vessels are an important site for the formation and actions of Ang-(1-7) (Santos *et al.* 2000, Santos & Ferreira 2007). Endothelial cells are not only involved in the production but also in the metabolism of this heptapeptide (Velez *et al.* 2012). The ACE2/Ang-(1-7)/Mas axis induces the release of vasodilators, including prostanoids, NO and endothelium-derived hyperpolarizing factor (Porsti *et al.* 1994, Brosnihan *et al.* 1996, Li *et al.* 1997, Muthalif *et al.* 1998, Iyer *et al.* 2000, Fernandes *et al.* 2001, Heitsch *et al.* 2001). Accordingly, Ang-(1-7) elicits relaxation in several vascular beds (see Santos *et al.* (2000) for review). In line with these observations, activation of endogenous ACE2 provokes reductions in arterial blood pressure of normal and hypertensive rats (Hernandez Prada *et al.* 2008). However, contradictory effects of Ang-(1-7) have been reported in human vessels. While Sasaki *et al.* (2001) reported vasodilation in the human forearm, Davie & McMurray (1999) did not observe any effect of Ang-(1-7) in the same territory in ACE inhibitor-treated patients.

Because plasma levels of Ang-(1-7) are increased during treatment with ACE inhibitors or AT₁ receptor blockers part of their effects might be dependent on Ang-(1-7) (Ferrario 2006). Indeed a role for Ang-(1-7) in mediating the decrease in blood pressure produced by treatment with losartan in normal rats has been proposed (Collister & Hendel 2003).

Several mechanisms are involved in the Ang-(1-7) vasodilatory effect, depending on the vessel diameter, the vascular regional bed, and the species. A complex interaction between Mas, BK B₂, AT₁R and AT₂R appears to be involved in mediating the Ang-(1-7) effects in some blood vessel preparations (Brosnihan *et al.* 1996, Gorelik *et al.* 1998, Soares de Moura *et al.* 2004).

Ang-(1-7) potentiates BK in several vascular beds and species. This BK-potentiating effect of Ang-(1-7) may contribute for its cardiovascular effects in normotensive (Paula *et al.* 1995, Oliveira *et al.* 1999, Greco *et al.* 2006) and hypertensive (Lima *et al.* 1997, Fernandes *et al.* 2001) rats. Several mechanisms have been proposed to explain the BK-potentiating activity of Ang-(1-7), including ACE inhibition since Ang-(1-7) is an ACE substrate (Li *et al.* 1997, Tom *et al.* 2001), allosteric changes in ACE (Erdos *et al.* 2002), and Mas-mediated changes in the BK signaling (Paula *et al.* 1995, Oliveira *et al.* 1999, Fernandes *et al.* 2001, Ferreira *et al.* 2001). In addition, vascular Ang-(1-7) actions could involve modulation of vascular effects of Ang II (Roks *et al.* 1999, Stegbauer *et al.* 2004).

Mas is involved in most of the known vascular effects of Ang-(1-7) and is present in human endothelial cells (Santos *et al.* 2005, Sampaio *et al.* 2007b). The endothelium-dependent relaxation induced by Ang-(1-7), AVE 0991 and CGEN-856S in mouse aortic rings is absent in vessels derived from Mas-deficient mice and is blocked by A-779 (Santos *et al.* 2003a, Lemos *et al.* 2005, Savergnini *et al.* 2010). A link between Mas and NO release was described by Pinheiro *et al.* (2004) and Sampaio *et al.* (2007b). Sampaio *et al.* (2007b) found that in both Mas-transfected Chinese hamster ovary (CHO) cells and human aortic endothelial cells (HAECs) Ang-(1-7) induces the release of NO through coordinated phosphorylation/dephosphorylation of eNOS. According to these authors, Ang-(1-7) induced phosphorylation of the stimulatory site Ser¹⁷⁷⁷ and dephosphorylation of the inhibitory site Thr⁴⁹⁵ (Sampaio *et al.* 2007b). This effect could involve the activation of the PI3K-AKT pathway. The Mas antagonist A-779 blocked all the effects of Ang-(1-7) on eNOS in both cell types. These *in vitro* observations are in keeping with the effects of Ang-(1-7) on endothelial function *in vivo* (Faria-Silva *et al.* 2005).

Renal actions of the ACE2/Ang-(1-7)/Mas axis

A number of evidences substantially support the important role of ACE2/Ang-(1-7)/Mas axis in renal function. The renal concentration of Ang-(1-7) and Ang II is comparable (Joyner *et al.* 2007), and it is possible to detect

Ang-(1-7) in the human urine. Interestingly, untreated patients with essential hypertension present decreased amounts of Ang-(1-7) in the urine when compared with healthy volunteers (Ferrario *et al.* 1998). Moreover, the enzymes involved in the formation of Ang-(1-7) are abundant in the kidney (Erdos & Skidgel 1990). By using mass spectrometry, Grobe *et al.* (2012) studied the distribution of the enzymatic machinery involved in Ang II metabolism within the mouse kidney. They reported that Ang-(1-7) and Ang-(1-4) were predominantly formed in the renal cortex, while Ang III was mainly produced in the renal medulla. The colocalization of Ang-(1-7) and Ang-(1-4) suggests that ACE2 and NEP are mainly found in the renal cortex. Indeed, in the kidney NEP plays a key role in the degradation of Ang-(1-7) to form its metabolite Ang-(1-4). Alike in the circulation, renal NEP also catalyzes the formation of Ang-(1-7) from Ang I or Ang-(1-9) (Allred *et al.* 2000, Chappell *et al.* 2001, Dilauro & Burns 2009, Pinheiro & Simoes 2012). In another recent study, which investigated the localization of the renal RAS components by immunohistochemistry, ACE2 was predominantly found along the proximal nephron (Pohl *et al.* 2010). Moreover, ACE2 was reported to be 20-fold more active in the mouse renal cortex than in cardiac tissue (Wysocki *et al.* 2006). Although most of the studies reported that ACE2 is predominantly expressed in the renal cortex, there are still some controversies regarding its localization since i) semiquantitative RT-PCR analysis revealed that ACE2 mRNA is found in all nephron segments – including the medulla with the exception of the medullary thick ascending limb of Henle's loop (mTAL); and ii) ACE2 protein was detected by western blot and immunohistochemistry in the outer medulla and inner medulla, besides the renal cortex (Li *et al.* 2005).

Recently, Pohl *et al.* (2010) studied the ability of the scavenger receptor megalin to modulate the expression of ACE and ACE2 in the brush border membrane of the proximal tubule. The authors observed that megalin-deficient mice had a higher immunofluorescence staining for ACE2 when compared with megalin-positive cell populations. By contrast, ACE staining was weaker in brush border membranes of megalin-deficient proximal tubular cells. This result suggests that megalin itself or megalin-related pathways regulate the expression of both isoforms in proximal tubular cells.

Mas seems to have a broad localization in the kidney. Alenina *et al.* (2008) reported that Mas mRNA is predominantly found in the renal cortex of male mice. Indeed, functional Mas is detected in proximal tubules,

as well as in the afferent arterioles, collecting ducts and the thick ascending limb of Henle and its expression is upregulated in renal ischemia (Ren *et al.* 2002, Gwathmey *et al.* 2010, Silveira *et al.* 2010, Zimmerman & Burns 2012).

Ang-(1-7) is known to induce an antiproliferative and protective effect by counterregulating the MAP kinase signaling induced by Ang II via AT₁ receptors (Sampaio *et al.* 2007a). Interestingly, Liu *et al.* (2012) recently reported that Ang-(1-7) induced ERK1/2 activation in glomerular mesangial cells via Mas. However, the authors demonstrated that, diverging from Ang II that induces ERK1/2 activation via NADPH oxidase activation or epidermal growth factor receptor (EGFR) transactivation, Ang-(1-7)/Mas leads to ERK1/2 phosphorylation in a cAMP/PKA-dependent manner (Liu *et al.* 2012), suggesting that, in these cells, Mas is coupled to Gs α . Rakusan *et al.* (2010) found that Mas deletion exacerbated renal hypertension in mice that was reversed with tempol or apocynin again suggesting a role in oxidative stress.

The Ang-(1-7) effects in the kidney are quite complex and controversial. A diuretic/natriuretic action of Ang-(1-7) due to inhibition of sodium reabsorption at the proximal tubule was proposed. In this context, Ang-(1-7) seems to limit transcellular sodium flux by modulating the activity of transporters via phospholipase A2 activation in tubular epithelial cells (Andreatta-van Leyen *et al.* 1993). Moreover, this heptapeptide appears to be a potent inhibitor of Na⁺-K⁺-ATPase activity in the renal cortex and in isolated convoluted proximal tubules (Handa *et al.* 1996, Lopez Ordieres *et al.* 1998, Burgelova *et al.* 2002, De Souza *et al.* 2004, Dilauro & Burns 2009, Pinheiro & Simoes 2012). This inhibitory effect is reversed by the AT₂ receptor antagonist PD 123319 in a dose-dependent manner in the inner cortex basolateral membrane from pig kidney. Interestingly, neither A-779 nor losartan affected this process, indicating that the inhibition of the Na⁺-K⁺-ATPase activity by Ang-(1-7) is mediated by AT₂ or another PD 123319-sensitive mechanism (De Souza *et al.* 2004). *In vitro* studies indicated that Ang-(1-7) reverses the stimulatory effect of Ang II on the Na⁺-ATPase activity in proximal tubule via Mas (Burgelova *et al.* 2002).

In contrast to that, an antidiuretic/antinatriuretic effect of Ang-(1-7) was observed in water-loaded rats (for review, see Joyner *et al.* (2008), Dilauro & Burns (2009) and Pinheiro & Simoes (2012)) and this effect seems to be at least partially mediated by Mas activation since A-779 blocks the Ang-(1-7) antidiuretic effect (Santos

et al. 1996). Simoes e Silva *et al.* (1998) reported that chronic administration of A-779 in both normotensive and SHR rats leads to natriuresis and diuresis. In addition, A-779 treatment in virgin female rats leads to increased urinary volume and decreased osmolality with no changes in water intake (Joyner *et al.* 2008). On the other hand, the Mas agonist AVE 0991 significantly reduced urinary volume (Pinheiro *et al.* 2004). All these data suggest that the diuretic/natriuretic effect observed in water-loaded animals are induced by the Mas activation. However, it is important to point out that other Ang receptors than Mas (e.g. AT₁ and AT₂) could also be involved in this process. A study on tubular bicarbonate transport implicated the AT₁ receptor in a biphasic response to Ang-(1-7) (Garcia & Garvin 1994).

The above-mentioned discrepancies between the Ang-(1-7) actions in the kidney may be explained by differences in experimental design (for example, *in vitro* vs *in vivo*), animal model (e.g. conscious vs anesthetized animals), Ang-(1-7) concentration, nephron segment, species, level of RAS activity, and water and salt status. Moreover, Ang-(1-7) can induce opposite effects in different physiological situations of an animal. For example in pregnant rats, Ang-(1-7) induces diuresis associated with downregulation of aquaporin-1 while in virgin females this heptapeptide leads to antidiuresis and upregulation of aquaporin-1 (Joyner *et al.* 2008). It is evident that the renal RAS depicts an intricate regulatory mechanism far more complex than expected. Nonetheless, further studies need to clarify the mechanisms underlying the renal actions of Ang-(1-7).

Besides its physiological relevance for kidney homeostasis, the ACE2/Ang-(1-7)/Mas axis also has an important and controversial role in renal diseases, acting as a renoprotective (Oudit *et al.* 2006, 2010, Soler *et al.* 2007, Pinheiro *et al.* 2009, Burns *et al.* 2010, Dilauro *et al.* 2010, Zhang *et al.* 2010, Giani *et al.* 2011, 2012, Liu *et al.* 2011b, Moon *et al.* 2011, Stegbauer *et al.* 2011, Zhong *et al.* 2011, Barroso *et al.* 2012, Kim *et al.* 2012, Nadarajah *et al.* 2012, Zhou *et al.* 2012) or a pro-inflammatory (Esteban *et al.* 2009, Velkoska *et al.* 2011) agent. Briefly, Pinheiro *et al.* (2009) reported that mice with genetic deletion of Mas developed renal dysfunction with an increase of glomerular tuft diameter and enhancement of fibronectin and collagen IV and III deposition, besides an increase of AT₁ and TGF- β expression. Moreover, Ang-(1-7) infusion counter-regulates Ang II to reduce glomerulosclerosis in experimental glomerulonephritis (Zhang *et al.* 2010). In addition, chronic inhibition of ACE2 with MLN-4760

in both healthy and diabetic mice led to glomerular injury and albuminuria (Soler *et al.* 2007). It is important to highlight that the ACE levels were notably increased in this study (Soler *et al.* 2007). Moreover, ACE2 was downregulated in the cortex of mice subjected to subtotal nephrectomy (Dilauro *et al.* 2010). Administration of recombinant ACE2 diminished fibrosis, i.e. glomerular mesangial matrix, smooth muscle actin and collagen III expression in diabetic AKITA mice (Oudit *et al.* 2010) and in wild-type mice infused with Ang II (Zhong *et al.* 2011). Accordingly, knockout mice for ACE2 infused with Ang II showed enhanced collagen I deposition and expression of genes related to fibrosis, such as smooth muscle actin, TGF- β , and procollagen I, probably through activation of ERK1/2 and enhancement of protein kinase C levels (Zhong *et al.* 2011). Taken together, these data suggest that renal pathogenesis is driven by a disruption in the ACE–ACE2 balance. Nonetheless, there are some studies suggesting a deleterious role for the renal ACE2/Ang-(1-7)/Mas axis that are conflicting with the above-mentioned studies. For example, Esteban *et al.* found that Mas knockout mice presented an attenuation of renal damage in a renal insufficiency model. The authors reported that Ang-(1-7) infusion led to NF- κ B activation and inflammation via Mas (Esteban *et al.* 2009). Tikellis *et al.* described that ACE2 knockout mice exhibit an increased albuminuria in a streptozotocin-induced diabetes model, but at the same time an attenuated expression of marker genes for diabetic nephropathy. Moreover, Velkoska *et al.* (2011) reported that Ang-(1-7) treatment of male Sprague–Dawley rats after subtotal nephrectomy induced deleterious cardiovascular effects as well as increasing levels of fibrosis when compared with controls. By contrast, no aggravation of renal injury produced by kidney ischemia–reperfusion was observed in Mas^{-/-} mice, and in the same model the Mas agonist AVE 0991 reduced renal injury (Barroso *et al.* 2012). Ang-(1-7) was also reported to stimulate (Burns *et al.* 2010) or to inhibit (Zhou *et al.* 2012) the epithelial-to-mesenchymal transformation in tubular cells.

These conflicting findings are probably due to differences in the models used and to cell-specific Ang-(1-7) signaling in the kidney, for example the dependency of each model on COX-2-mediated events, which can be differentially modulated by Ang-(1-7) (Albrecht 2007, Menon *et al.* 2007), and highlight the need for further *in vivo* studies related to the RAS and its novel axis in kidney disease.

Metabolic actions of the ACE2/Ang-(1-7)/Mas axis

The existence of local RAS has already been reported in the endocrine and exocrine pancreas (Chappell *et al.* 1991, Leung *et al.* 1997, 1999), as well as in the adipose tissue (Schling *et al.* 1999). The components of these local RAS are highly regulated by food intake. High sugar diet increased the expression levels of angiotensinogen, ACE and AT₁ in pancreas (Lupi *et al.* 2006). Similarly, a diet rich in fat or sugar increased the concentration of ACE2 and Ang-(1-7) in adipose tissue (Gupte *et al.* 2008, Coelho *et al.* 2010). Therefore, the RAS is considered a potential target for treating the metabolic syndrome, which is characterized by obesity, insulin resistance, hypertension, dyslipidemia, and other symptoms (Grundey *et al.* 2004).

The observation that insulin resistance is frequently associated with cardiovascular impairments suggests interplay between the RAS and insulin. Such a relationship was prompted by clinical trials (Hansson *et al.* 1999, Brenner *et al.* 2001, Yusuf *et al.* 2001, Dahlof *et al.* 2002) and experimental studies (Oliveira *et al.* 2002, Furuhashi *et al.* 2004, Lupi *et al.* 2006) where the overall observation was an improvement in hyperglycemia by inhibiting the RAS either with ACE inhibitors or AT₁ antagonists. Moreover, it has already been shown that the upregulation of ACE2 also improves hyperglycemia in diabetic rats (Bindom *et al.* 2010). Therefore, it is clear that, together with BK, Ang-(1-7) has an important antihyperglycemic effect while Ang II acts in the opposite way. Indeed, many studies have reported that Ang-(1-7) attenuates the manifestations of the metabolic syndrome, increases glucose uptake and protects cells against the oxidative stress that can induce insulin resistance (Santos *et al.* 2008, Giani *et al.* 2009, Liu *et al.* 2011a).

The molecular mechanisms underlying the positive regulation of insulin promoted by Ang-(1-7) are now being revealed. It has been reported that Ang-(1-7) and insulin have some common downstream signaling effectors in HAEC (Sampaio *et al.* 2007b) and in the hearts (Giani *et al.* 2007). Ang-(1-7) induces the phosphorylation of the insulin downstream effectors PI3K and AKT via Mas in HAEC (Giani *et al.* 2007, Sampaio *et al.* 2007b) and IRS-1 and JAK2 via AT₁ receptor in hearts (Giani *et al.* 2007). Moreover, Ang-(1-7)/Mas negatively regulates Ang II/AT₁ signaling in HAEC by promoting dephosphorylation of c-Src and ERK1/2 and inhibition of NADPH oxidase activity (Sampaio *et al.* 2007a). Recently, our group used a phosphoproteome approach to study Ang-(1-7) signaling in human endothelial cells. This study revealed novel downstream components of Ang-(1-7)/Mas signaling and

provided additional evidence for an interplay between insulin and Ang-(1-7) networks (Verano-Braga *et al.* 2012).

Lipid metabolism is also regulated by Ang-(1-7). Mas knockout mice on FVB/N background had impairments in lipid metabolism, leading to dyslipidemia, lower glucose tolerance and insulin sensitivity, hyperinsulinemia, hyperleptinemia, lower adiponectin secretion, decreased glucose uptake and increased abdominal fat mass when compared with the wild-type phenotype (Santos *et al.* 2008). On the other hand, despite the normal food intake, TGR(A1-7)3292 animals with increased plasmatic levels of Ang-(1-7) showed reduced fat mass and decreased triglycerides and cholesterol levels. In addition to that, the expression levels of adiponectin and adipose lipid-binding protein (AP2) were increased while there was a remarkable decrease in the angiotensinogen expression in these animals. Adiponectin is a key adipokine that regulates insulin sensitivity and tissue inflammation and its plasmatic level is inversely proportional to body fat content, and AP2 is an important protein in adipose tissue metabolism involved in fatty acid esterification (Santos *et al.* 2010). In the same way, ACE knockout mice presented reduced fat mass due to an increased lipid metabolism and energy expenditure as a consequence of higher expression levels of key genes involved in the hydrolysis of lipids into free fatty acids (lipoprotein lipase (LPL)), translocation of fatty acids to the mitochondria (carnitine palmitoyltransferase 1 (CPT-1)), and β -oxidation inside mitochondria and peroxisomes (long-chain acyl-CoA dehydrogenase (LCAD); Jayasooriya *et al.* 2008).

Thus, considering that comparable results were obtained using different animal models and technical approaches, we believe that there is enough evidence proving a key role of the ACE2/Ang-(1-7)/Mas axis in the regulation of carbohydrate and lipid metabolism.

Ang-(1-7) as an AT₁ receptor antagonist

Mahon *et al.* (1994) described that Ang-(1-7) can antagonize the pressor effect of Ang II in anesthetized rats. This was achieved with very high doses of Ang-(1-7). Lately, Rowe *et al.* (1995) reported that Ang-(1-7) binds to AT₁ receptors. Again, this was observed at high concentrations of Ang-(1-7) ($\geq 10^{-6}$ mol/l). However, Gironacci *et al.* (1999) reported that Ang-(1-7) may compete for the binding of AT₁ receptors with high affinity ($K_i = 8.0 \pm 3.2$ nM) in kidney slices. Moreover, Ang-(1-7), which was without effect on basal $[Ca^{2+}]_i$, reduced Ang II- and Ang IV-dependent $[Ca^{2+}]_i$ increases in mesangial cells (Chansel *et al.* 2001). In common, most of

these studies used pharmacological concentrations of Ang-(1-7), suggesting that, in this condition, Ang-(1-7) could compete for AT₁ receptors (Oudot *et al.* 2005).

At physiological concentrations, Ang-(1-7) is an endogenous modulator of the AT₁-mediated Ang II responses. This phenomenon is clearly observed at the intracellular level. Ang-(1-7) antagonized the Ang II-induced activation of protein kinase C and ERK1/2 in vascular smooth muscle cells (VSMCs; Zhu *et al.* 2002). This peptide also significantly attenuated the Ang II-induced reactive oxygen species generation, c-Src and ERK1/2 activation, and stimulated SHP-2 in CHO cells transfected with Mas and in HAEC (Sampaio *et al.* 2007a). Tallant & Clark (2003) demonstrated that Ang-(1-7) inhibits Ang II stimulation of ERK1/2 in cultured rat aortic VSMCs through a prostacyclin-mediated production of cAMP and activation of cAMP-dependent protein kinase. In addition, Ang II-induced ERK1/2 phosphorylation was also inhibited by Ang-(1-7) in rat cardiomyocytes. This effect was abolished by transfection of cells with an antisense oligonucleotide to Mas (Tallant *et al.* 2005). In proximal tubular cells, Ang II-stimulated phosphorylation of three MAP kinases (p38, ERK1/2 and c-Jun) was also inhibited by Ang-(1-7), an effect that was also completely blocked by the Mas antagonist A-779 (Su *et al.* 2006). Furthermore, a Mas-dependent inhibition of Ang II-induced EGFR transactivation by Ang-(1-7) has also been demonstrated in rat VSMCs (Akhtar *et al.* 2012).

Additionally, it could be possible that Ang-(1-7) modulates Ang II effects at the molecular level during AT₁ mRNA synthesis and/or translation. Actually, AT₁ receptor expression appears to be downregulated in CHO cells stably transfected with the AT_{1a} receptor pretreated with 1 or 10 μ M Ang-(1-7) (Clark *et al.* 2001b). This effect was also observed in rat aortic VSMCs (Clark *et al.* 2001a) and in the cortical tubulointerstitial area of the kidney (Clark *et al.* 2003). However, Neves *et al.* (2000) have found that Ang-(1-7) upregulates the mRNA expression of the AT₁ receptor in VSMCs. A similar upregulation was reported by Canals *et al.* (2006) in cells overexpressing Mas.

Several studies have also described a direct physical interaction with a possible antagonistic effect between Mas and the AT₁ receptor (Kostenis *et al.* 2005, Santos *et al.* 2007). Future studies are obviously necessary to clarify the nature of the interaction between both receptors.

Ang-(1-7) and the AT₂ receptor

Ang-(1-7) has a very low affinity for AT₂ receptors (Rowe *et al.* 1995). Indeed, many of the Ang-(1-7) effects were not

influenced by AT₂ receptor antagonists (Brosnihan *et al.* 1996, Santos *et al.* 2000, Tallant *et al.* 2005, Dharmani *et al.* 2007, Silva *et al.* 2007). However, in certain circumstances and in some tissues AT₂ receptors appear to be involved in Ang-(1-7) actions. For instance, in isolated mouse hearts treated with the AT₂ antagonist PD 123319, Ang-(1-7) produced an increase in the perfusion pressure that could not be attributed to interaction with AT₁ or Mas receptors (Castro *et al.* 2005). In this preparation, blockade of AT₁ receptors unmasked a vasodilator effect of Ang-(1-7) at a subpicomolar concentration, which was Mas dependent. Another intriguing observation was reported by Walters *et al.* (2005) in candesartan-treated SHR. In these animals, Ang-(1-7) produced a substantial decrease in blood pressure, which was not modified by the Ang-(1-7) receptor antagonist A-779 but was fully blocked by the AT₂ antagonist PD 123319. There are few other reports in which an effect of Ang-(1-7) was blocked or attenuated by PD 123319 but not by A-779 (De Souza *et al.* 2004, Lara *et al.* 2006, Pereyra-Alfonso *et al.* 2007).

In sharp contrast with these observations are several studies describing a complete or, less frequently, partial inhibition of Ang-(1-7) effects with A-779 (Santos *et al.* 2000, Ferreira *et al.* 2001, Santos *et al.* 2005, Ferrario 2006, Gallagher *et al.* 2006). In some studies a partial blockade was obtained with the AT₂ antagonist PD 123319 when a partial inhibition was also seen with A-779 and the B₂ receptor antagonist Hoe 140. Likewise, some BK effects are attenuated by PD 123319 (Bergaya *et al.* 2004) and some AT₂-mediated effects are attenuated by B₂ receptor blockade (Munk *et al.* 2007). These observations illustrate the intricate relationship between Ang and kinin receptors, suggesting intracellular interactions via common signaling pathways or heterodimerization. The mechanisms of such interaction are currently only elusive. However, physical interactions between Mas and AT₂ or B₂ receptors in selected tissues should be considered as explanation for some of the puzzling observations with the use of receptor antagonists.

Concluding remarks

The complexity of the RAS is far beyond what we could suspect few years ago. New elements have recently been added to the system (ACE2, Ang-(1-7), Mas, (Pro)renin receptor) (Ferreira & Santos 2005, Ferrario 2006, Santos & Ferreira 2007, Sihn *et al.* 2010) and have completely changed its perception. It is now generally accepted that the RAS is dual and that, besides the well known mainly deleterious arm (ACE/Ang II/AT₁), there is a second

beneficial axis consisting of ACE2, Ang-(1-7), and Mas. The evidence summarized in this review clearly shows a participation of this new RAS axis in the regulation of blood pressure and metabolism, and, even more importantly, in the pathogenesis of, at least, cardiovascular, renal, and metabolic diseases. Therefore, the novel concept of the dual RAS is invigorating the development of new cardiovascular drugs activating the beneficial arm of the RAS (Ferreira *et al.* 2012).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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