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

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 hypothalamic–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 solitarii (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.
2000, Ocaranza et al. 2006), which is subsequently cleaved to generate Ang-(1–7) through ACE and neutral endopeptidase 24.11 (NEP) hydrolysis (Rice et al. 2004). However, apparently the generation of Ang-(1–7) directly from Ang II through the cleavage of the C-terminal amino acid phenylalanine is physiologically and biochemically more relevant (Vickers et al. 2002). Therefore, ACE2 plays a pivotal role in the body as an endogenous regulator of the RAS, once it can degrade Ang II, a vasoconstrictor/proliferative peptide, and produce Ang-(1–7), a vasodilator/antiproliferative peptide. It should be pointed out, however, that other enzymes can form Ang-(1–7) from Ang I or Ang II, including prolylendopeptidase, prolylcarboxypeptidase, thimet oligopeptidase, and NEP (Chappell et al. 1998, Stanziola et al. 1999, Campbell et al. 2004; Fig. 1). In particular NEP may contribute substantially to the plasma levels of the heptapeptide (Chappell et al. 2000).

Another important step to establish the relevance of Ang-(1–7) was achieved with the identification of its receptor, the G protein-coupled receptor Mas (Santos et al. 2003b). This finding confirmed previous evidence obtained in functional studies (Campagnole-Santos et al. 1992, Santos et al. 2000) and with the use of the Ang-(1–7) antagonists A-779 (Ambuhl et al. 1994, Santos et al. 1994) and D-Pro7-Ang-(1–7) (Santos et al. 2003a), suggesting that this peptide exerts its actions through binding to a receptor distinct from AT1 and AT2 receptors.

It is now accepted that the ACE2/Ang-(1–7)/Mas axis is able to counteract most of the deleterious actions of the ACE/Ang II/AT1 receptor axis especially in pathological conditions (Ferreira & Santos 2005; Fig. 2). However, the role of Ang-(1–7) is not limited to its counterregulatory action. Indeed, genetic deletion of Mas produces an extremely rich phenotype which includes cardiac dysfunction (Santos et al. 2006), increased blood pressure (genetic background dependent) (Xu et al. 2008), decreased baroreflex function (de Moura et al. 2010), endothelial dysfunction (Xu et al. 2008), reduced reproductive function, increased thrombogenesis (Fraga-Silva et al. 2008) and, depending on genetic background, marked changes in lipid and glucose metabolism leading to a metabolic syndrome like state (Santos et al. 2006; Fig. 3).

Most of these observations are corroborated by data obtained with ACE2-deficient mice (Crackower et al. 2002, Yamamoto et al. 2006, Jin et al. 2012).

In this review, we will briefly highlight recent findings concerning the cardiovascular, renal, and metabolic roles of the ACE2/Ang-(1–7)/Mas axis. Furthermore, we will address the potential interactions of Ang-(1–7) and Mas with AT1 and AT2 receptors.

**Cardiac actions of the ACE2/Ang-(1–7)/Mas axis**

The presence and synthesis of RAS components in the heart suggest that locally produced bioactive Ang peptides modulate cardiac function and structure (Grinstein & Young 1992, Dostal & Baker 1999). Ang-(1–7) is among the RAS components present in hearts. The localization and

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**Figure 1**
Detailed representation of the renin–angiotensin system cascade. The metabolic pathways involved in the generation of the main products of this system are focused in the insert. ACE, angiotensin-converting enzyme; Ang, angiotensin; Aog, angiotensinogen; AMP, aminopeptidase; AT1, Ang II type 1 receptor; AT2, Ang II type 2 receptor; Mas, Ang-(1–7) receptor; D-Amp, dipeptidyl-aminopeptidase; IRAP, insulin regulated aminopeptidase; PCP, prolylcarboxypeptidase; PEP, prolylendopeptidase; NEP, neutral endopeptidase 24.11; (P)RR, (pro)renin receptor.
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.
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 vasodilation 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 (De Mello 2004). Additionally, Ang-(1–7) decreased total K+ apparently involving activation of the sodium pump (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 antiarrhythmic effect of Ang-(1–7) (Ferreira et al. 2012). This peptide also improved posts ischemic 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 posts ischemic 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 (HPβCD) 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 HPβCD/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 (Grove 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 prostanoioids, NO and endothelium-derived hyperpolarizing factor (Porsli 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 volunteeres (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,

http://joe.endocrinology-journals.org
DOI: 10.1530/JOE-12-0341
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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 activation or epidermal growth factor receptor that induces ERK1/2 activation via NADPH oxidase the authors demonstrated that, diverging from Ang II activation in glomerular mesangial cells via Mas. However, recently reported that Ang-(1–7) induced ERK1/2 activation 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).

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-beta, 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-kB 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 (Grundy 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 lip-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 I (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) (≥ 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 (Kᵢ = 8.0 ± 3.2 nM) in kidney slices. Moreover, Ang-(1–7), which was without effect on basal [Ca²⁺]ᵢ, reduced Ang II- and Ang IV-dependent [Ca²⁺]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 AT1R receptor pretreated with 1 or 10 μM 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.

Funding

This work was supported by CAPES/DAAD (PROBRAL).

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Received in final form 18 October 2012
Accepted 22 October 2012
Accepted Preprint published online 22 October 2012