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ACE2/ANG-(1–7)/Mas pathway in the brain: the axis of good

Ping Xu, Srinivas Sriramula, and Eric Lazartigues

Department of Pharmacology and Experimental Therapeutics and Cardiovascular Center of Excellence, Louisiana State University Health Sciences Center, New Orleans, Louisiana

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Xu P, Sriramula S, Lazartigues E. ACE2/ANG-(1–7)/Mas pathway in the brain: the axis of good. *Am J Physiol Regul Integr Comp Physiol* 300: R804–R817, 2011. First published December 22, 2010; doi:10.1152/ajpregu.00222.2010.—The last decade has seen the discovery of several new components of the renin-angiotensin system (RAS). Among them, angiotensin converting enzyme-2 (ACE2) and the Mas receptor have forced a reevaluation of the original cascade and led to the emergence of a new arm of the RAS: the ACE2/ANG-(1–7)/Mas axis. Accordingly, the new system is now seen as a balance between a provasoconstrictor, profibrotic, progrowth axis (ACE/ANG-II/AT_1 receptor) and a provasodilatory, antifibrotic, antigrowth arm (ACE2/ANG-(1–7)/Mas receptor). Already, this simplistic vision is evolving and new components are branching out upstream [ANG-(1–12) and (pro)renin receptor] and downstream (angiotensin-IV and other angiotensin peptides) of the classical cascade. In this review, we will summarize the role of the ACE2/ANG-(1–7)/Mas receptor, focusing on the central nervous system with respect to cardiovascular diseases such as hypertension, chronic heart failure, and stroke, as well as neurological diseases. In addition, we will discuss the new pharmacological (antagonists, agonists, activators) and genomic (knockout and transgenic animals) tools that are currently available. Finally, we will review the latest data regarding the various signaling pathways downstream of the Mas receptor.

renin-angiotensin system

THE RENIN-ANGIOTENSIN SYSTEM (RAS) is a peptide hormone system composed of various enzymes, inactive and active peptides, which altogether play an important role in cardiovascular physiology, by regulating blood pressure (BP) and volume homeostasis. Classically, angiotensinogen (AGT) produced in the liver, is hydrolyzed by renin from the juxtaglomerular cells of the kidney to produce the decapeptide ANG-I, which is then converted by angiotensin converting enzyme (ACE) into the biologically active octapeptide ANG II. AGT is the protein precursor of the RAS main actor ANG II. Cleavage of AGT by the rate-limiting enzyme renin produces an inactive decapeptide, ANG I, acting mostly as a substrate for ANG II, which is generated by the proteolytic ablation of the two COOH-terminal amino acids of ANG I by the mainly endothelium-associated ACE (137). Despite being discovered more than 100 years ago, the RAS still represents a key target for the treatment of various cardiovascular diseases. Originally, the RAS was considered to be an endocrine system with circulating ANG II as its functional effector hormone. However, in the recent decade with the advent of new molecular techniques there have been significant changes in our view of this system, and a new axis, ACE2/ANG-(1–7)/Mas receptor, was established. In the year 2000, ACE2, a new member of the ACE family, was identified by two independent groups. ACE2 can cleave the decapeptide ANG I to generate the inactive ANG-(1–9) peptide, which then can be converted to the vasodilatory peptide ANG-(1–7) by ACE or other peptidases. ACE2 can also directly metabolize ANG II to generate ANG-(1–7). ANG-(1–7), the main product of ANG II degradation by ACE2, has opposite properties to that of ANG II. By acting through the receptor Mas, ANG-(1–7) promotes vasodilation, antiproliferation, and antihypertrophy (43, 129). Accumulating evidence indicates that by cleaving ANG II into ANG-(1–7), ACE2 may play a pivotal role in counterbalancing the vasoconstrictive actions of the ACE/ANG II/AT_1 receptor axis and may be beneficial for the cardiovascular system.

Some of the peripheral therapeutic effects of the novel ACE2/ANG-(1–7)/Mas receptor axis were recently reviewed by Ferreira et al. (45). In this review, we will focus on the role of this system in the central nervous system (CNS) and its participation in central BP regulation and various cardiovascular diseases linked to an overactive brain RAS.

Historical Perspective

Classical textbooks emphasize that there are two key producing enzymes in the RAS: renin and ACE. Renin was the first component of the RAS to be discovered following the observation by Tigersted in 1898 that rabbit renal extracts had a pressor activity (140). Further clarification of the mechanism of action of renin was delayed by the difficulty in obtaining a reliable model of renovascular hypertension. This was achieved in 1934, when Goldblatt et al. (65) described an increase in systolic BP following clipping of the renal arteries,

Address for reprint requests and other correspondence: E. Lazartigues, Louisiana State Univ. Health Sciences Center, School of Medicine, Dept. of Pharmacology and Experimental Therapeutics, 1901 Perdido St., New Orleans, LA 70112 (e-mail: elazar@lsuhsc.edu).

thus opening the road for further characterization of renin substrate and product. Two independent groups led by Dr. Eduardo Braun-Menéndez in Argentina and Dr. Irving Page in the United States reported the discovery of the renin product that they respectively called "hypertensin" (11) and "angiotonin" (104). Soon after, the nomenclature for the renin substrate was changed to AGT (105), and an agreement was later reached between the two groups for a common nomenclature of the renin product under the name "angiotensin" (12).

ACE was discovered in the plasma by Skeggs et al. in 1956. The conversion of the inactive ANG I to the vasoconstrictor ANG II was thought to take place in the plasma. However, in 1967, Ng and Vane showed that the plasma ACE was too slow to account for the conversion of ANG I to ANG II in vivo and that rapid conversion actually occurs through the pulmonary circulation (100).

Several ANG II receptors have been cloned over the years (21, 154). The ANG II receptor type I (AT_1) is the primary receptor that mediates most of the effects of ANG II, including vasoconstriction, water intake, and aldosterone secretion. In rodents, AT_1 receptors are subdivided into AT_{1A} and AT_{1B}. The latter has been suggested to regulate water intake (27), while the AT_{1A} subtype would be responsible for the other main properties of ANG II. A type II (AT_2) receptor was also cloned (154) and is thought to play a major role in fetal development, although recent data seem to contradict this assumption (165). In addition, recent data on AT_2 receptors have suggested compensatory properties in the brain and other tissues, such as decreasing the nocturnal arterial BP in rats (59). Both type I and type II receptors bind to ANG II with similar affinity but display different functions. Other receptors include the AT_4 subtype, which was originally discovered in the brain as a binding site for ANG IV (71) and is involved in memory processes and Alzheimer's disease. More recently, presence of a non-AT_1/non-AT_2 receptor was suggested within the CNS (82). This new binding site displayed high affinity for ANG I, II, and III, but lesser affinity for smaller angiotensin fragments and other neuropeptides and therefore could be a clearance receptor for degradation of ANG II from the extracellular milieu in the brain (82).

Discovery and Evolution of the Brain RAS

Observations of normal or even lower plasma renin and ACE activities in animal models of hypertension, combined with clinical data showing beneficial effects of ACE inhibitors in hypertensive patients with low plasma ANG II levels (99) suggested the existence of a nonsystemic RAS. In the following years, all the major components of this system were found to coexist in several tissues, including the brain, heart, adipose tissue, vasculature (106), kidney, and adrenal gland (72).

Long before this recognition, it was already known that infusion of ANG II into the brain could increase BP (8) and central injection of purified ANG II near the hypothalamus resulted in a drinking response (37, 58), suggesting the presence of specific receptors in this tissue. More important was the first evidence that renin is present within the brain, thereby providing the enzymatic synthesis of local angiotensin production within this tissue (49). At that time, the existence of the brain RAS was first postulated when Ganten et al. (58) identified renin-like activity in the CNS.

The main feature of this system is its distinction from the other local or tissue RAS, since it is physically separated from the endocrine RAS by the presence of the blood-brain barrier, thus preventing the diffusion of ANG II from the circulation into the brain (130). However, the existence of areas lacking a blood-brain barrier, called circumventricular organs (CVOs), challenged this presumption and has been responsible for a long debate surrounding the existence of the brain RAS. Indeed, in mammals, there are eight of these CVOs, located in the proximity of the 3rd and 4th ventricles: the vascular organ of the lamina terminalis, the subfornical organ, the median eminence, the intermediate and the posterior lobes of the hypophysis, the subcommissural organ, the pineal gland, and the area postrema (34, 80). Most of these CVOs have fenestrated capillaries allowing molecules of large molecular weight to cross back and forth between the circulation and the cerebrospinal fluid; therefore circulating ANG II may still produce some effects inside the brain (136). The difficulty in detecting significant amounts of renin in the CNS and the presence of the CVOs has sparked some debate over the existence of ANG II generation in the brain (57). Nevertheless, expression of local ANG II was later found in the hypothalamic paraventricular nucleus (PVN), supraoptic nucleus, CVOs, and nucleus of the tractus solitarii (NTS) neuronal cell bodies (90). In addition, AGT synthesis in astrocytes and its secretion into the interstitial space and cerebrospinal fluid was shown to be the major source of substrate for brain ANG II formation (29). The controversy surrounding the existence of the brain RAS slowly eroded with more evidence showing renin expression in the CNS. It is now accepted that secreted prorenin and nonsecreted renin are present in the brain of rodents and humans (86), and their overexpression results in a hypertensive phenotype, confirming the pivotal role of this system in the regulation of BP and the development of hypertension. Interestingly, recent data have also confirmed the presence of intracellular renin in the CNS, encoded by a different gene (i.e., renin-b) and functionally capable of increasing BP (85).

The ACE2/ANG-(1–7)/Mas Axis in the Brain

ANG-(1–7) synthesis and metabolism. The physiological presence of ANG-(1–7) was first detected in human blood (132) and later, in the dog (123) and rat brain (131). ANG-(1–7) was shown to be present as an endogenous constituent of the brain, in areas including the hypothalamus, medulla oblongata, and amygdala, as well as in adrenal glands and plasma of normal rats (19). However, the enzymatic cascades leading to the generation of this peptide are learned later. We now know of three different pathways to produce ANG-(1–7), as reviewed in details by Karamyan and Speth (82). First, directly from ANG I by prolyl-endopeptidase or neutral endopeptidase (neprilysin), which cleave the bond at residues Pro^7-Phe^8 (156); second, directly from ANG II by ACE2, prolyl-carboxypeptidase or prolyl-endopeptidase; and third, indirectly, ACE2 converts ANG I to ANG-(1–9). ANG-(1–7) is then produced by ACE cleavage of the dipeptide phenylalanine-histamine from ANG-(1–9) (145) or by neprilysin (118). Several other enzymes can also participate in each of these three pathways (82).

However, it has recently been shown that, in the hypertensive rat heart, the majority of the ANG-(1–7) formed results

from the degradation of ANG II by ACE2 (144). It is conceivable that the other pathways might be activated or inhibited in specific pathological conditions. It is likely that the synthesis of ANG-(1–7) is taking place mostly in the extracellular space since ACE2 is a transmembrane protein with its catalytic site located outside the cell (70). However, because ACE2 conserves its activity following shedding by ADAM17, one can speculate that endocytosis of the secreted enzyme could lead to formation of the heptapeptide inside the cell. This hypothesis is consistent with our observation of the enzyme in the cytoplasm of neurons in the mouse brain (33) and the existence of an intracellular RAS in neurons (66) (Fig. 1). After synthesis, ANG-(1–7) can be metabolized into ANG-(1–5) by ACE (20) or ANG-(1–4) by neprilysin (2). Interestingly, ANG-(1–7) can inhibit the proteolytic function of ACE by binding with ACE at the COOH-terminal domain, thus promoting bradykinin function (142).

ANG-(1–7) function. To our knowledge, the first study involving ANG-(1–7) peptide in the CNS was from Fitzsimons (50), showing that unlike ANG II, ANG-(1–7) has no dipsogenic effect when injected into the rat brain. Immunocytochemical studies have localized ANG-(1–7) in neuronal cell bodies and fiber tracts of magnocellular nuclei in the rat hypothalamus (9, 83). It is well recognized that blood vessels are an important site for the formation and biological actions of ANG-(1–7) (73). In endothelial cells, ANG-(1–7) stimulates prostaglandin release (79), increases the release of nitric oxide (NO) (111), augments the metabolic actions of bradykinin via inhibition of ACE activity (1), and inhibits smooth muscle cell growth (54). In vitro experiments have shown that ANG-(1–7) has a potent vasopressin (AVP) and prostaglandin-releasing activity and promotes neuronal activity in the hypothalamus and medulla (52), although the effect on AVP release appears to be much less compared with ANG II (113).

Soon after the identification of ANG-(1–7) in the brain (123, 131), the peptide was reported to produce depressor responses when administered in the NTS and dorsal motor nucleus of the vagus nerve (15). The NTS serves as the first brain relay for the information originating from the baroreceptors located in the carotid arteries and the aortic arch, while the dorsal motor nucleus of the vagus nerve is one of the nuclei controlling parasympathetic tone (18). Unlike in the brain stem, administration of ANG-(1–7) in the lateral ventricles failed to alter mean arterial BP or heart rate (HR) but resulted in an increase in cardiac baroreflex sensitivity (16). A similar response was also observed following peripheral infusion of ANG-(1–7) in spontaneously hypertensive (SH) rats (7) or when low doses of the peptide were combined with low doses of bradykinin (10), suggesting a synergistic effect between the two peptides. Simultaneous infusion of ANG-(1–7) and bradykinin at subeffective rates into the brain resulted in a significant increase in baroreflex sensitivity, suggesting that centrally these two peptides can interact to modulate baroreflex control of HR. Interestingly, the ANG-(1–7) permissive effect is only targeting the bradycardic component of the reflex (16), when BP rises following administration of the pressor agent and activation of the central regions results in the increase of vagal tone. It has also been suggested that the opposing actions of endogenous ANG II and ANG-(1–7) in the NTS contribute to baroreflex function in response to increases in mean arterial BP in young rats (120). The mechanism by which ANG-(1–7) regulates baroreflex sensitivity maybe derived from its ability to reduce sympathetic tone and modulate the local effects of norepinephrine (NE) in the brain. Treatment with ANG-(1–7) inhibited ANG I- and ANG II-mediated facilitation of NE release in isolated kidneys of SH stroke-prone and Wistar-Kyoto rats (138). Gironacci et al. have shown that, while the heptapeptide had no effect on NE uptake and catabolism (60), it could decrease NE release (62) and ANG II-mediated NE release (63). Moreover, this mechanism was mediated by NO and blocked by both AT_2 and bradykinin B_2 receptor antagonists. Very recently, these authors extended their findings by

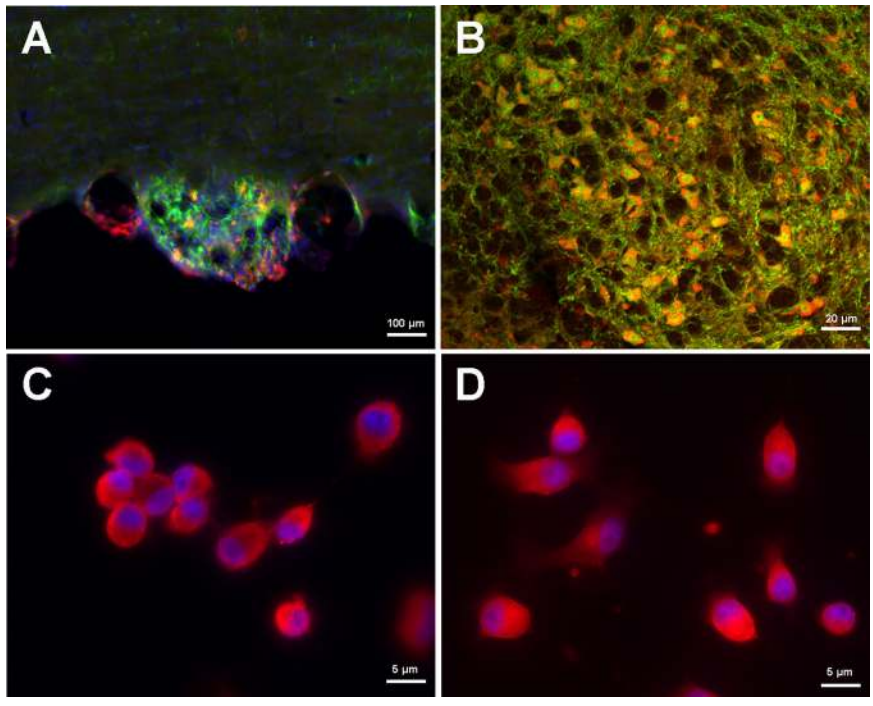

Fig. 1. Endogenous angiotensin converting enzyme 2 (ACE2) and Mas receptor immunostaining in mouse brain sections and neuronal cell cultures. Mouse subfornical organ (*A*) and rostral ventrolateral medulla (*B*) double stained for ACE2 (red) and a neuronal marker (NeuN; green) are shown. Yellow staining is indicative of the presence of ACE2 in neurons. Neuro2A cells (mouse neuroblastoma) were stained for endogenous ACE2 (*C*) and the Mas receptor (*D*) confirming the presence of these renin-angiotensin system (RAS) components in neurons.

showing that ANG-(1–7) induces a decrease in tyrosine hydroxylase expression, the rate-limiting enzyme in catecholamine biosynthesis. This decrease was also blocked by an AT_2 receptor antagonist and not by an AT_1 or Mas receptor antagonist (91). This observation reveals that ANG-(1–7) downregulation of tyrosine hydroxylase activity and expression centrally may decrease brain catecholaminergic activity; however, failure of the Mas antagonist to block this decrease suggests that ANG-(1–7) may bind with another receptor (52, 119).

While ANG-(1–7) and ANG II have opposite effects on baroreflex function following injection into the dorsal medulla, both peptides have similar responses when injected in the rostral (RVLM) or caudal (CVLM) ventrolateral medulla suggesting differential effects in certain brain areas (31). Administration of ANG II into the RVLM has been shown to activate neurons in vitro (88) and to increase BP in anesthetized and conscious rats (51, 52). However, the activated RVLM neurons are different from the pacemaker noradrenergic presympathetic cells described in this region (88). Similarly, ANG-(1–7) produces a pressor response, generally associated with a tachycardia, that can be blocked by the selective antagonist D-Ala^7-ANG-(1–7) (A-779) (51) and which is enhanced by hemorrhage (89). When injected in the CVLM, both ANG II and ANG-(1–7) produce a decrease in mean arterial BP, although the signaling pathways activated seem to be different. In Wistar rats, the ANG-(1–7)-mediated BP reduction is attenuated by L-nitro-arginine methyl ester (L-NAME) and neuronal NO synthase blockers, while they are ineffective on ANG II responses (3). In addition, the heptapeptide increases L-glutamate levels in the CVLM, while taurine release is reduced (152). Furthermore, although CVLM injection of ANG-(1–7) depresses both femoral and renal vascular resistances, ANG II only affects the kidneys' vascular bed (47). Microinjection of ANG II and ANG-(1–7) into the CVLM produces similar decreases in BP in rats with renovascular hypertension (2K1C) and in sham animals. Importantly, the weak reflex bradycardia observed in 2K1C rats can be improved following microinjection of A-779 into the CVLM, while losartan does not enhance the baroreflex sensitivity, suggesting that ANG-(1–7) at the CVLM may contribute to the low sensitivity of the baroreflex control of HR in hypertensive rats (17).

Additional effects of brain ANG-(1–7) include modulation of the BP and HR circadian rhythms (133) and enhancement of long-term potentiation in the CA1 region of the hippocampus (74). Central administration of ANG-(1–7) increases cerebral blood flow (121), bradykinin levels (93), NO release, and endothelial NO synthase expression (166), which is beneficial in cerebrovascular diseases. In a recent study, central administration of ANG-(1–7) reduced neurological deficits and infarct size in a rat model of ischemic stroke, demonstrating cerebroprotective properties of this peptide during ischemic stroke (95).

Agonists and Antagonists

Potent peptidic antagonists of the ANG-(1–7) receptor have been generated by substituting the C-proline with a D-alanine, to form D-Ala^7-ANG-(1–7), also called A-779 (4, 124), or with a D-proline, to obtain D-Pro^7-ANG-(1–7) (128). Utilization of these antagonists has been useful in unmasking specific effects of ANG-(1–7). Indeed, although chronic infusion of ANG-(1–7) failed to modify the development of Goldblatt's 2K1C hypertension and renal function, chronic infusion of A-779 resulted in a higher increase in mean arterial BP and a reduction in renal plasma flow (13). Moreover, A-779 was critical in establishing that ANG-(1–7) acts through a specific binding site, independent of the AT_1 and AT_2 receptors (5, 6, 52, 107, 124, 134). Interestingly, blockade of ANG-(1–7) receptors in the PVN by A-779 uncovered an unusual role for the heptapeptide in the maintenance of sympathetic nerve activity. Indeed, microinjection of ANG-(1–7) into the PVN increases renal sympathetic nerve activity, showing an excitatory action for the heptapeptide on PVN neurons, and this effect can be blocked by its selective antagonist A-779 (134).

Recently, the use of the D-Pro^7-ANG-(1–7) antagonist has led to the identification of a new binding site for ANG-(1–7) in the rat aorta, which could not be blocked by A-779 (135). The existence of this second receptor for ANG-(1–7) remains to be confirmed.

The first nonpeptidic and orally active agonist of ANG-(1–7) (5-formyl-4-methoxy-2-phenyl-1-{(4-[2-ethyl-aminocarbonyl-sulfonamido-5-isobutyl-3-thienyl]-phenyl)-methyl}-imidiazole, also known as AVE0991) was generated by Dr. Holger Heitsch's group at Aventis Pharmaceuticals, to clarify the role of ANG-(1–7) in potentiating bradykinin responses (155). Like ANG-(1–7), this compound is capable of producing NO release (155), thus improving endothelial function (38) and is a ligand for the Mas receptor (87, 110). While the central effects of this compound have not been investigated, Wessel et al. (153) reported that SH rats treated with this compound have a significantly lower increase in BP during the night when the animals are active. In addition, while AVE0991 did not affect the baroreflex gain, the activation of this compensatory mechanism was less (153), suggesting that in ANG-(1–7) agonist-treated SH rats, baroreceptors were less stimulated than in control hypertensive animals.

Transgenic Models

TGR(A1–7) 3292 transgenic rats, exhibiting chronic production of ANG-(1–7), were engineered by using a fusion protein methodology. Although the transgene is driven by a cytomegalovirus promoter, its expression appears restricted to the testes (127). In this model, the male gonads are functioning like a biological infusion pump, as evidenced by the ~2.5-fold increase in systemic ANG-(1–7) levels, and therefore could modify the activity of local RAS in various organs. While these rats are less sensitive to induction of cardiac hypertrophy by isoproterenol (127) and show a decrease in vascular resistance for several organs, including the brain, there has been no study addressing the central implications of ANG-(1–7) overexpression in this model. Santos et al. (127) reported a significant increase in HR for which they speculated a potential interaction of the peptide at the CVO level. It would be particularly interesting to see whether these animals show signs of altered sympathetic drive and baroreflex function in normal and pathophysiologic conditions.

ACE2 Gene and Protein

While the existence of angiotensin peptides resulting from the degradation of ANG II was already known 40 years ago, the importance of these peptides has been debated for decades,

in part due to the incertitude regarding the enzyme responsible for their formation. Despite the numerous observations showing its benefits on baroreceptor reflex, cardiac, and vascular functions, the role of ANG-(1–7) remained underappreciated until the discovery in 2000 of a new carboxypeptidase responsible for the conversion of ANG II into the vasodilatory heptapeptide (32, 141). The discovery of this new enzyme, named ACE2, due to its homology with ACE (40% identity and 61% similarity), from human heart failure ventricle (32) and human lymphoma cDNA libraries (141), was followed in 2003 by the identification of a specific receptor (i.e., Mas) for ANG-(1–7) (129). Together, these critical findings gave a new impetus for the understanding of the role of this new arm of the RAS, which is now known as the ACE2/ANG-(1–7)/Mas receptor axis.

ACE2 is a glycoprotein of 120 kDa, expressed as a transmembrane protein but also exists in a soluble, truncated form, lacking the transmembrane and cytosolic domains, but conserving its activity (141). This metalloprotease contains a single zinc-binding domain and conserves other critical residues typical of the ACE family. The protein sequence consists of 805 amino acids, including a potential 17-amino acid NH_2-terminal signal peptide sequence and a putative COOH-terminal membrane anchor. Functionally, ACE2 acts as a carboxypeptidase to cleave the COOH-terminal leucyl residue from ANG I, thus producing Ang-(1–9). However, so far there has been no confirmation that this reaction takes place and is physiologically relevant in vivo. More importantly, the enzyme is also able to hydrolyze ANG II to produce ANG-(1–7) and release phenylalanine (141). ACE2 shows 400-fold higher affinity for ANG II than for ANG I (147), making it the main substrate of the enzyme. In vitro, ACE2 has also been reported to cleave des-Arg-bradykinin, apelin fragments, and neurotensin but not bradykinin or any of the 15 other vasoactive and hormonal peptides tested (147).

Regulation of ACE2 Expression

Originally, immunohistochemistry showed ACE2 protein predominantly in the endothelium of various vessels in the heart and kidney and in renal tubular epithelium (32). In addition, expression of ACE2 mRNA was found in colon, small intestine, ovary, testis, prostate, heart, placenta, liver, skeletal muscle, and pancreas with the highest levels of expression in lung and kidney (141), and it is now evident that most tissues express this carboxypeptidase.

In the brain, ACE2 was reported to be widely distributed, in the cytoplasm of neuronal cell bodies but not in glial cells (33, 81). This is actually surprising since it is thought to be a transmembrane protein with most of its structure on the extracellular side. This observation suggests that a significant pool of ACE2 might be stored inside cytoplasmic vesicles. Whether the cytoplasmic ACE2 might play a role within the intracellular RAS remains to be determined. In vitro, other groups have also observed ACE2 expression in astroglial cells (55). Brain ACE2 activity was also reported to be the highest in the hypothalamus of C57BL/6 mice (36). In the subfornical organ, an area lacking the blood-brain barrier and sensitive to blood-borne ANG II, ACE2 immunostaining was significantly increased in the brain of transgenic mice overexpressing neuron-specific AT_{1A} receptors (NSE-AT_{1A}) and chronically hypertensive mice overexpressing human AGT and renin genes (R^+A^+) (33). This increase was not present in other hypothalamic regions, such as the PVN, suggesting a nucleus-specific regulation of the enzyme. Furthermore, in the RVLM, Yamazato et al. (162) observed a significant reduction of ACE2 protein levels in SH rats, while in R^+A^+, the enzyme activity, but not expression (158), was impaired. In both cases, ACE2 gene therapy was associated with a decrease in BP. In neonatal rat cerebellar or medullary astrocytes, ANG II reduced ACE2 mRNA and protein expression, and this inhibition could be prevented by an AT_1 receptor antagonist but not by an AT_2 antagonist. On the other hand, ANG-(1–7) did not affect ACE2 mRNA, but prevented the ANG II-mediated reduction in ACE2 mRNA. Restoration of the inhibitory effect was achieved by addition of A779, confirming the involvement of the heptatpeptide (55). More recently, Kar et al. (81) showed that in chronic heart-failure rabbits, ACE2 expression was reduced in the RVLM, while ACE was upregulated, a situation that could be reversed by exercise training. The observation that ACE2 can be downregulated by ANG II or AT_1 receptors (55, 81, 158) constitutes a novel positive feed-forward system within the brain, and clearly more work is needed to understand the various mechanisms leading to the alteration of ACE2 gene expression. Alternatively, ACE2 could also affect the expression of angiotensin receptors. Our group previously showed that, both in vitro and in vivo, ACE2 overexpression led to a downregulation of AT_1 receptors in a neuronal cell line and in the subfornical organ of normotensive C57BL/6 mice (40). These data were recently confirmed in hypertensive mice and extended by the observations of a concomitant increase in both AT_2-to-AT_1 and Mas-to-AT_1 receptor ratios in the dorsal and ventral medulla (39). Therefore, not only ACE2 expression can be modulated by the classical RAS, but the enzyme can also alter the system, suggesting the existence of a mutual regulation between the two arms of the RAS. The main challenge is now to determine the ideal conditions for which ACE2 would exert its maximal inhibitory effect on the classical RAS.

ACE2 Inhibitors and Agonists

Only a small number of antagonists have been generated to target the carboxypeptidase, probably due to the overwhelming reports showing that ACE2 expression might be beneficial in various diseases, thus limiting the therapeutic interest of an enzyme inhibitor. Originally, these antagonists were designed to reduce the binding of the severe acute respiratory syndrome (SARS) coronavirus, known to use ACE2 as a functional receptor, with the enzyme and prevent the infection of pulmonary cells and neurons (143). However, the consensus has changed, and it now appears that ACE2 might also be beneficial in preventing the progression of SARS into acute respiratory distress syndrome (78). Accordingly, the focus has shifted toward identifying compounds that may stimulate ACE2 activity or mimic the effects of endogenous ACE2. While the following compounds have been extensively used to increase ACE2 expression in peripheral tissues, very limited data are available regarding their ability to alter ACE2 activity in the brain.

The first antagonist generated, MLN-4760 (renamed GL-1001), allowed inhibition of ACE2 in the picomolar range,

while conserving a very good selectivity vs. ACE and carboxypeptidase A, as confirmed by X-ray crystallography (26). It was later shown that this antagonist binds with the active site of the enzyme and as such, modulates catalysis and substrate (143). While numerous groups have used this antagonist at the periphery, there has been only one study evaluating its effects in the brain. Following administration into the NTS of anesthetized Sprague-Dawley rats, MLN-4760 was reported to produce a long-lasting reduction in mean arterial BP, while not affecting HR (30). Moreover, the ACE2 antagonist impaired the reflex bradycardia resulting from the systemic administration of phenylephrine, consistent with the detrimental and beneficial effects on baroreflex sensitivity of ANG II and ANG-(1–7), respectively. Finally, in anesthetized C57BL/6 mice, we also observed that intracerebroventricular administration of MLN-4760 (4–20 μg) produced a dose-dependent increase in mean BP (Lazartigues E, unpublished data), suggesting that ACE2 and ANG-(1–7) might be involved in the maintenance of basal BP.

Using an in silico molecular docking approach, another compound, *N*-(2-aminoethyl)-1 aziridine-ethanamine, was identified among ~140,000 small molecules, as a potential ACE2 inhibitor with an IC_{50} in the micromolar range. This compound was shown to be effective in blocking the SARS coronavirus spike protein-mediated cell fusion (77), but its effects in the CNS have not been investigated.

The only peptidic and commercially available antagonist, DX600, was identified through selection of constrained peptide libraries by phage display (76). The synthesized peptide is a potent inhibitor of ACE2 activity, with a K_i of 2.8 nM. It has been widely used for in vitro studies aimed at clarifying the signaling pathways activated by ACE2, but its effects in the brain remain to be determined. In a comparative study, it was used in conjunction with SELDI-TOF mass spectrometry to highlight a predominant role of ACE2 activity in the hypothalamus (36).

Following virtual screening of chemical libraries, based on the crystal structure of ACE2, two compounds were identified, a xanthenone and resorcinolnaphthalein, as potential activators of ACE2 (75). Interestingly, the xanthenone was effective in reducing BP following both acute and chronic peripheral administration in SH rats. Additional experiments in the setting of pulmonary hypertension also revealed that xanthenone exhibits properties beyond the activation of ACE2, notably the ability to increase both ACE2 and Mas mRNA expressions (46). However, it is not clear whether xanthenone is active only locally or whether it can affect ACE2 activity in multiple tissues. It would be most interesting to determine its impact on baroreflex and autonomic function following peripheral or central administration. In a recent study, intravenous administration of diminizene aceturate, a known antiprotozoal drug used in humans, caused a transient and dose-dependent decrease in mean arterial BP in Wistar-Kyoto and SH rats (64). Although diminizene aceturate decreased BP in both strains of rats, it was shown that its efficacy was significantly higher in SH rats. Further studies are needed to understand the role of this drug on the hypertensive response.

More recently, a soluble and highly glycosylated recombinant human ACE2 was developed and shown to be effective in preventing ANG II-dependent hypertension and diabetic nephropathy (103, 157). However, ANG-(1–7) was not found to be necessary for the reduction of hypertension using recombinant human ACE2 and which appeared to be only mediated by a reduction in ANG II levels (157). Of interest was also the lack of increased ACE2 activity in cardiac and renal tissues while it was enhanced in the plasma. This raises the question of whether this approach is only beneficial for hypertensive patients presenting elevated ANG II plasma levels. Additional studies are clearly needed to determine whether patients with neurogenic hypertension might benefit from this novel therapeutic approach.

Knockout and Transgenic Animal Models

At least four different ACE2 knockout mouse models have been generated by gene targeting on both C57BL/6 and 129/SvEv backgrounds (24, 68, 102, 161). While the 129/SvEv background did not show any alteration of cardiovascular function, controversial data have been reported on the C57BL/6 background, with evidence for severe cardiac contractility defects (24), cardiac dysfunction following pressure overload (161), and elevated baseline BP (68). For a detailed analysis of the various phenotypes, see Ref. 69. Despite these discrepancies, all models exhibited exacerbated responses to ANG II.

The mechanism(s) by which hypertension develops in ACE2 gene deficiency may be derived from peripheral endothelial dysfunction and/or alteration of central neuronal regulation. In the periphery, it was reported that ACE2-deficient mice exhibit impaired endothelium-dependent relaxation (92), while centrally, ACE2 gene deletion resulted in impaired baroreflex and autonomic functions (Fig. 2) (159).

To understand the role of ACE2 in the central regulation of BP and the development of hypertension, we previously developed several transgenic mouse models overexpressing this enzyme specifically in the CNS (39, 158, 159). Targeting ACE2 expression selectively on neurons by using a synapsin promoter in syn-hACE2 transgenic mice did not affect baseline hemodynamic parameters but altered the balance between ANG II and ANG-(1–7) levels in the brain, in favor of the vasodilatory peptide (39). However, expression of the enzyme throughout the brain was able to abate the development of neurogenic hypertension after 2 wk of peripheral infusion of a subpressor dose of ANG II. Indeed, while control littermates exhibited ANG II-mediated impairments in baroreflex function and vagal tone, syn-hACE2 mice remained protected, partly through enhanced expression of NO synthases in the brain and modulation of ANG receptors. Interestingly, neurogenic hypertension could be achieved in syn-hACE2 following concomitant infusion of ANG II and the A-779 antagonist, supporting a pivotal role for ANG-(1–7). To address the potential benefits of ACE2 gene therapy in the maintenance of hypertension, we used a double transgenic mouse (R^+A^+) overexpressing both human renin and AGT genes (97). In these chronically hypertensive R^+A^+ mice, we observed a significant reduction of ACE2 activity in the brain and impaired baroreflex sensitivity (158). Breeding of the syn-hACE2 onto the R^+A^+ background allowed us to generate a triple transgenic mouse (SARA) in which we could assess the effects of ACE2 reintroduction. Interestingly, these SARA mice, although still hypertensive, showed a reduced BP level and improved baroreflex and autonomic functions. Others have

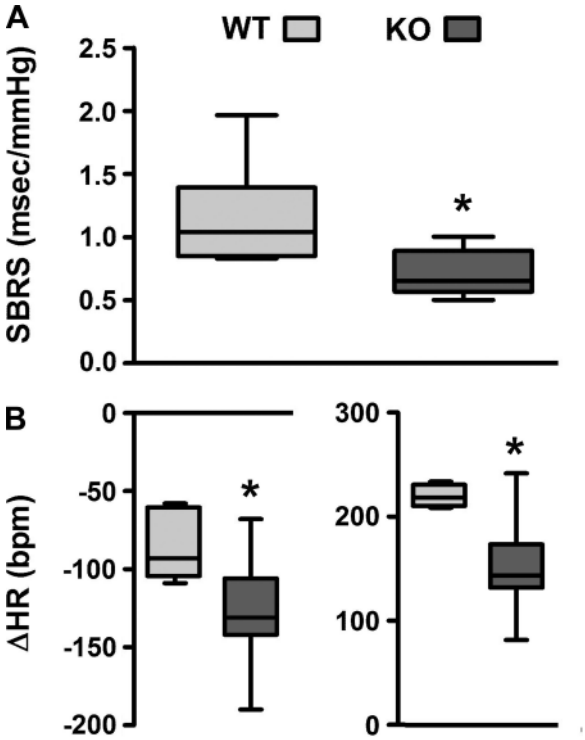

Fig. 2. Impaired spontaneous baroreflex sensitivity (SBRS) and autonomic function in ACE2$^{-/y}$ knockout (KO) mice. SBRS (*A*) was significantly decreased in KO mice compared with the wild-type (WT) littermates. Meanwhile, sympathetic tone (*B; left*) was significantly increased and parasympathetic tone (*B; right*) was significantly decreased in the ACE2-deficient mice compared with WT, as evidenced by the bradycardic and tachycardic responses to propranolol and atropine, respectively. HR, heart rate; bpm, beats/min. *$P < 0.05$ vs. WT.

shown that a similar reduction in BP could also be achieved in SH rats following RVLM administration of a lentivirus encoding ACE2 (162). In SARA mice, the persistence of some degree of hypertension can be explained by the high systemic ANG II levels that could not be corrected by central overexpression of ACE2. However, the enhanced water intake observed in R^+A^+ could be normalized in SARA mice, illustrating the powerful therapeutic potential of ACE2 gene therapy.

Finally, a similar transgenic model was also generated by overexpressing ACE2 selectively on vascular smooth muscle cells in a stroke-prone SH rat (117). These animals also showed a reduction of hypertension as well as improvement of endothelial function. While the specific effects of ACE2 overexpression on the brain vessels has not yet been investigated in these rats, it would be interesting to determine the impact on central BP regulatory mechanisms, but also in other pathologies such as chronic heart failure and stroke.

Mas Receptor

Mas was originally described as a protooncogene, due to its ability to induce tumorigenicity in nude mice (164). The human *Mas* gene was mapped to chromosome 6 (6q24–6q27), within a region frequently rearranged in malignant cells (115). The protein has seven hydrophobic transmembrane domains, while the NH_2- and COOH-terminal ends are hydrophilic. It shares a strong sequence similarity with the G protein-coupled receptor subfamily of hormone-receptor proteins (112). In 2003, Santos et al. (129) identified ANG-(1–7) as a ligand for the Mas receptor, establishing the ACE2/ANG-(1–7)/Mas axis as a new arm of the RAS.

Mas expression in mice was found in the heart, kidney, lung, liver, spleen, tongue, and skeletal muscle (98, 148). In the heart, low levels of *Mas* transcripts were detected in cardiomyocytes and much more in the endothelium of coronaries. Similarly, *Mas* expression was also observed in brain endothelial cells derived from rat cerebral resistance vessels (84). While Mas mRNA expression was originally thought to be restricted to the hippocampus, cortex, and olfactory bulb (98, 163), later development of specific antibodies extended these observations to other brain structures. A dense Mas immunoreactive staining was observed in cardiovascular-related areas, from the medulla to the forebrain, such as the NTS, RVLM, CVLM, inferior olive, parvo- and magnocellular portions of the PVN, supraoptic nucleus, and lateral preoptic area, shown in several previous studies as sites for the action of ANG-(1–7) in the brain (125). Moreover, at the cellular level, Mas was predominantly present in neurons, as evidenced by colocalization of immunostaining for the neuronal marker, Neu-N, and the Mas receptor antibody (6). However, it was recently reported that astrocytes located in the RVLM of Wistar rats could respond to ANG-(1–7) stimulation, leading to increases in intracellular Ca^{2+}, while neurons were nonresponsive to the heptapeptide (67). Interestingly, this response was prevented by administration of A-779, suggesting the participation of Mas receptors. While an impairment of intracellular Ca^{2+} increases was also evidenced in SH rats, suggesting a potential role in hypertension, it is unknown how activation of Mas signaling in astrocytes could affect sympathetic tone and modulate BP regulation.

Mas Knockout Mice

Targeted deletion of the genomic region coding for the first 253 amino acids of Mas, including six transmembrane domains, led to a loss of Mas expression (150). The homozygous *Mas*-deficient mice on the mixed 129xC57BL/6 genetic background are healthy, grow normally, and show no alteration of baseline BP and HR in both genders. However, significant differences appear on both HR variability and BP variability, two relevant predictors of arterial hypertension and cardiovascular diseases in humans. *Mas*-deficient females show a strong reduction of HR variability, while an increase in BP variability is observed in males, thus shifting the autonomic balance toward increased sympathetic tone in both sexes (151).

Like for the ACE2 knockout mice (69), the genetic background is known to dramatically influence the phenotype. Following backcrossing on a FVBN background, for seven generations, *Mas*-deficient mice exhibit signs of mild hypertension (160). In addition, *Mas*-deficient mice on a mixed FVBN and C57BL/6 background have impaired endothelial function and decreased NO production (114). ANG-(1–7)-mediated relaxation of isolated mesenteric arteries is equally impaired in both wild-type mice pretreated with A-779 and *Mas*-deficient mice (108). Moreover, the response to the endothelium-dependent vasorelaxant, bradykinin and acetylcholine, is similarly inhibited.

It is not clear whether any of these phenotypic alterations are mediated by the brain RAS since there has been no study

focusing in BP regulation and CNS in this model. The only data available are related to learning, memory, behavior, and synaptic plasticity. In an early study, Walther et al. (150) showed that *Mas* knockout mice exhibited enhanced long-term potentiation in the dentate gyrus, and elevated anxiety as evidenced by an increase in swimming speed when placed in a Morris water maze. More recently, the same group showed that ANG-(1–7) enhanced long-term potentiation when injected in the hippocampus and that this response was similarly impaired by A-779 and in *Mas*-deficient mice (74). Although these two studies may seem contradictory at first glance, it appears that the nuance is in the methodology used to achieve synaptic plasticity. Similarly, in the amygdala, ANG II was shown to increase the amplitude of field potentials in wild-type mice, while they were decreased by the same peptide in *Mas* knockout mice (149). Interestingly, these data suggest an interaction between both Mas and the AT_1 receptor to form functional heterodimers, a concept recently reviewed by Lyngsø et al. (94). An interaction between AT_1 and Mas receptors is also supported by observations in the kidney and vascular smooth muscle cells that ANG-(1–7) reduces AT_1 receptors (22, 23).

Agonists

The Mas agonists are one of the latest sets of tools developed to study the ACE2/ANG-(1–7)/Mas receptor axis, and no data are currently available regarding the potential effects of these drugs on CNS functions.

AVE0991 was the first nonpeptidic and orally active analog of ANG-(1–7) developed (155). It was demonstrated to be efficient in improving endothelial function (38), promoting cardioprotection (35, 44), and reducing hypertension (153) in rats. In the latter study, the authors reported a reduction of the nocturnal rise in BP, characteristic of rodents, and a significant reduction in baroreflex activation. However, the gain of the reflex was not altered, suggesting that the improvement might be related to a peripheral effect on BP and/or endothelial function rather than a central effect on baroreflex controlling centers.

More recently, using a computerized method aimed at identifying ligands for G protein-coupled receptors, two new compounds, CGEN-856 and CGEN-857, were identified as agonists of the Mas receptor. According to the data released by the company manufacturing these compounds, the lead peptide, CGEN-856, would induce relaxation of rodent aortas via Mas receptor activation and through a NO-mediated pathway. Additional information suggests a beneficial role in vivo on cardiac remodeling antihypertensive effects as well as cardiac and renal antifibrotic properties.

ACE2/ANG-(1–7)/Mas Downstream Signaling

Classical RAS signaling pathways (i.e., ANG II-mediated) in the brain have been well studied (for review see Refs. 25 and 146). The downstream signaling pathways of ACE2/ANG-(1–7)/Mas axis within the CNS involves intrinsic signaling molecules to induce vasoprotective actions by counterregulating the ACE/ANG II/AT_1 receptor axis (Fig. 3). We now know that binding of ANG II to the AT_1 receptor results in the activation of G_q-mediated phosphoinositide hydrolysis, which in turn

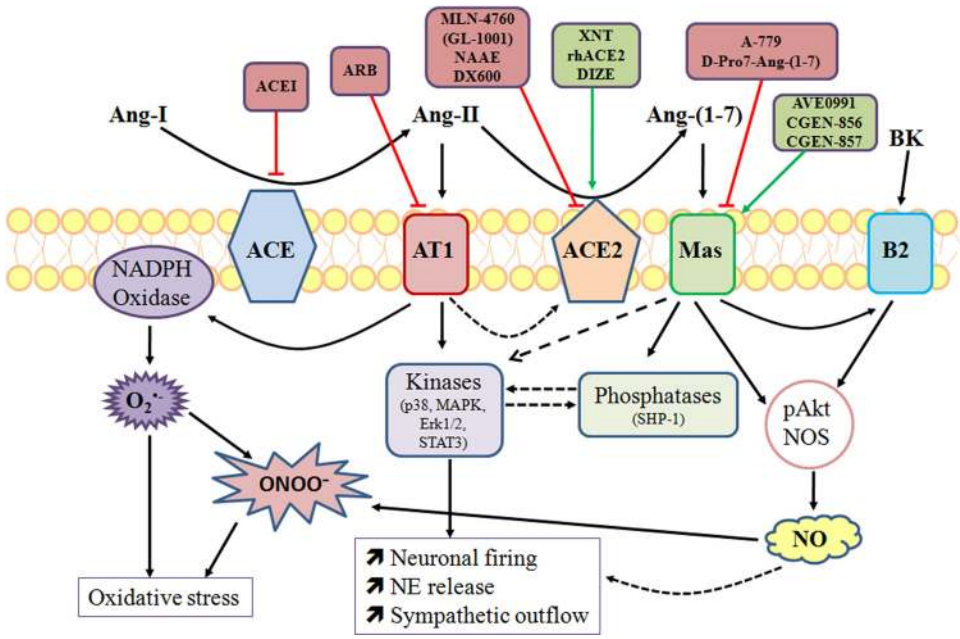

Fig. 3. Proposed ACE2/ANG-(1–7)/Mas signaling pathways in the central nervous system. ANG II is produced by ACE from ANG I and cleaved by ACE2 to form ANG-(1–7). ANG II binding to AT_1 receptors activate MAPK kinase, p38, Erk1/2, and this effect can be attenuated by ANG-(1–7) activation of the Mas receptor. STAT3 can be stimulated by both ANG II and ANG-(1–7). Following activation by the Mas receptor, Src homology 2-containing protein-tyrosine phosphatase-1 (SHP-1) inhibits MAP kinases activity. Kinases and phosphatases signaling exert a mutual inhibitory effect on each other. In the central nervous system, kinase activity determines neuronal firing, norepinephrine (NE) release, and sympathetic outflow. AT_1 receptor-mediated activation of NADPH oxidase leads to the formation of superoxide ($O_2^{\cdot-}$) acting with nitric oxide (NO) to form peroxynitrite ($ONOO^-$). NO release can result from the activation of both Mas and B_2 receptors via Akt phosphorylation (pAkt). Several agonists (green arrows) and antagonists (red lines) have been developed for the various components of this system. ARB, angiotensin type 1 receptor blocker; MLN-4760, antagonist GL-1001; NAAE, *N*-(2-aminoethyl)-1 aziridine-ethanamine; XNT, xanthenone; DIZE, diminazene aceturate; CGEN-856 and CGEN-857, agonists of the Mas receptor; BK, large-conductance Ca^{2+}-activated K^+ channel; NOS, nitric oxide (NO) synthase. Solid arrows, stimulation pathways; dashed lines, inhibitory pathways.

increases intracellular Ca^{2+} preceding the activation of protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). These signaling proteins are responsible for the inhibition of K^+ currents and activation of Ca^{2+} currents, ultimately leading to increased neuronal firing, which could lead to increased sympathetic outflow. Additionally, PKC can also activate the NAD(P)H oxidase, resulting in the formation of reactive oxygen species that have been involved in the development and maintenance of hypertension (169). In parallel, phospholipase C is activating a Ras/Raf/MAPK pathway responsible for the phosphorylation of c-jun and c-fos transcription factors, promoting the upregulation of genes involved in the synthesis and transport of NE in neurons.

Although signaling pathways downstream of the ANG-(1–7) receptor are not well characterized, the heptapeptide is generally thought to oppose the ANG II-mediated cascades (116). Among differences between the two peptides, ANG-(1–7) is not able to induce Ca^{2+} release (42, 73) and does not produce a dipsogenic effect (50). However, ANG-(1–7) is also capable of activating its own set of signaling molecules (41, 126).

The major downstream effector resulting from ANG-(1–7) receptor activation is NO. In the brain, the first evidence of a link between ANG-(1–7) and NO was from the observation of colocalization of the heptapeptide with NO synthase in neurons of the supraoptic nucleus and PVN (14). Focusing on the same brain region, Gironacci et al. (62) showed that NE release was impaired in hypothalamic preparations following administration of ANG-(1–7) and this effect could be reversed by L-NAME. Interestingly, the authors reported that the inhibitory effect on NE release could be blocked by both A-779 and an AT_2 receptor blocker, suggesting that several receptors are involved in this regulation. They later extended these results by showing that the bradykinin B_2 receptor was also involved and activated a cGMP/PKG pathway leading to NO release (61). Because NO is a diffusible gas, its presence in the brain could originate from different cell types, including neurons, endothelium, vascular smooth muscle cells, platelets, astrocytes, and glia. The ANG-(1–7)-induced NO release could be blocked by A-779, although not always completely (73), in various cell types and prevented in cells lacking Mas (39, 53, 62, 122), leaving no doubt for the critical role of this receptor. In human endothelial cells, constitutively expressing the Mas receptor, ANG-(1–7) activation of a PI3 kinase/Akt/protein kinase B pathway leads to long-lasting endothelial NO synthase (eNOS) phosphorylation of Ser^{1177} (122). In vivo, ANG-(1–7) stimulated NO release and upregulated eNOS expression in ischemic tissues following focal cerebral ischemia/reperfusion in rat models (166). Similarly, we reported that ACE2 overexpression resulted in increased NOS (both endothelial and neuronal) and NO levels in the cerebrospinal fluid of mice (39). Increased ACE2 expression on neurons resulted in enhanced eNOS phosphorylation of Ser^{1177}, while phosphorylation of Thr^{495}, representing inactive eNOS, was reduced. Moreover, brain AT_2-to-AT_1 and Mas-to-AT_1 receptor ratios were significantly increased in transgenic mice, suggesting that both AT_2 and Mas receptors may mediate the NO release.

It is well known that ANG II stimulates multiple kinases pathways (96). Treatment of vascular smooth muscle cells with ANG II increases MAPK p38 and Erk1/2 activities, and these responses could be reduced by pretreatment with ANG-(1–7) (56). Moreover, ANG-(1–7) also blocked ANG II-mediated reduction in ACE2 mRNA, supporting the concept of a reciprocal inhibition between the two RAS axes. Furthermore, the beneficial effects of ANG-(1–7) could be prevented by sodium vanadate and okadaic acid, suggesting that tyrosine phosphatases and serine-threonine phosphatases are activated by the vasodilatory peptide (56).

On the other hand, in mouse bone marrow-derived dendritic cells, ANG-(1–7) alone induced Erk1/2 phosphorylation, and an even greater response was observed when ANG II was coincubated (101). This synergistic effect was blocked by A-779, suggesting that the ANG-(1–7) receptor played a major part in this response. However, whether the ANG-(1–7)-mediated Erk1/2 phosphorylation is dependent on this particular cell type remains to be determined.

Several studies have reported that NO can react with locally produced superoxide ($O_2^{\cdot -}$) to form the cytotoxic peroxynitrite. While ANG II has been demonstrated to be a major player in the formation of $O_2^{\cdot -}$ in the brain and to participate in the development and maintenance of hypertension (168, 169), ANG-(1–7) itself can produce low levels of $O_2^{\cdot -}$ (73). However, the consensus is that the ANG-(1–7) NO/$O_2^{\cdot -}$-releasing profile might preserve endothelial function.

In addition, ACE2, either by reducing ANG II levels or by promoting ANG-(1–7)-mediated activation of downstream signaling, has also been shown to reduce oxidative stress in human endothelial cells (167). Particularly, in this study, the authors showed that ACE2 overexpression could prevent the ANG II-mediated upregulation of $p22^{phox}$, a major subunit of NAD(P)H oxidase.

A close and complex relationship has been described between ANG-(1–7) and bradykinin (for review see Ref. 126). For instance, the heptapeptide is known to inhibit ACE activity and thus favor an increase in bradykinin levels (28). As mentioned previously, bradykinin B_2 receptors are involved in the ANG-(1–7) inhibitory effect on NE release in the hypothalamus (62). Moreover, subeffective doses of ANG-(1–7) and bradykinin produced a synergistic effect on baroreflex sensitivity, while higher doses of each individual peptide were required to induce similar increases in gain (10). More recently, in rats undergoing medial cerebral artery occlusion to produce cerebral ischemia, ANG-(1–7) infusion was shown to markedly enhance bradykinin levels and to increase B_2 receptor mRNA and protein expression (93).

Several other signaling molecules and peptides have been reported to be affected by ANG-(1–7), including arachidonic acid, prostaglandins, AVP, and endothelium-derived hyperpolarizing factor (41, 126).

Perspectives and Significance

Far from the rigid and simplistic structure presented in textbooks, our present view of the RAS incorporating the ACE2/ANG-(1–7)/Mas axis is only part of the big picture, and it should only be considered as a temporary view, mostly dependent on our ability and determination to identify additional components. Already, data have emerged showing that additional elements play critical roles upstream and downstream of ANG II. Emphasizing the therapeutic importance of these new RAS members, phase I clinical trials have been completed (109), and the pharmaceutical industry has embarked on developing compounds targeting the nontraditional

components of the RAS (recombinant human ACE2, ANG-(1–7) agonists, prorenin receptor blockers). ANG-(1–7) is not the end of the story. Indeed, its cleavage by aminopeptidase A leads to ANG-(3–7), which has been shown to promote dopamine and GABA release in the striatum (139) and increase BP in the RVLM (48), and both observations could be important in the treatment of Parkinson's disease and hypertension, respectively. In the brain, like in the periphery, the RAS is involved in physiological functions beyond our current understanding, and keeping an open mind is our better chance to find cures and treatments for cardiovascular and other pathologies.

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DISCLOSURES

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