REVIEW

Angiotensin-converting enzyme 2 in the brain: properties and future directions

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Abstract

Angiotensin (Ang)-converting enzyme (ACE) 2 cleaves Ang-II into the vasodilator peptide Ang-(1–7), thus acting as a pivotal element in balancing the local effects of these peptides. ACE2 has been identified in various tissues and is supposed to be a modulator of cardiovascular function. Decreases in ACE2 expression and activity have been reported in models of hypertension, heart failure, atherosclerosis, diabetic nephropathy and others. In addition, the expression level and/or activity are affected by other renin–angiotensin system components (e.g., ACE and AT1 receptors). Local inhibition or global deletion of brain ACE2 induces a reduction in baroreflex sensitivity. Moreover, ACE2-null mice have been shown to exhibit either blood pressure or cardiac dysfunction phenotypes. On the other hand, over-expression of ACE2 exerts protective effects in local tissues, including the brain. In this review, we will first summarize the major findings linking ACE2 to cardiovascular function in the periphery then focus on recent discoveries related to ACE2 in the CNS. Finally, we will unveil new tools designed to address the importance of central ACE2 in various diseases, and discuss the potential for this carboxypeptidase as a new target in the treatment of hypertension and other cardiovascular diseases.

Keywords: angiotensin-converting enzyme 2, blood pressure, brain renin–angiotensin system, gene therapy.

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It is well established that the renin–angiotensin system (RAS) is an important factor in the regulation of blood pressure (BP). Classically, angiotensinogen (AGT) is hydrolyzed by renin to form the decapeptide Angiotensin (Ang)-I which is then converted by angiotensin-converting enzyme (ACE) into the biologically active peptide Ang-II. Ang-II leads to elevations in BP by promoting vasoconstriction, renal sodium and water reabsorption, increasing cardiac output, sympathetic tone, arginine vasopressin release, and stimulating the sensation of thirst in the CNS (Reid *et al.* 1978; Phillips 1987). While Ang-II can interact with both AT1 and AT2 receptor subtypes (Table 1), these effects of Ang-II are mediated mainly by AT1 receptors (AT1R). The ACE/Ang-II/AT1R axis has long been thought to be the main path for the RAS in controlling the regulation of cardiovascular function. Therefore, pharmacological inhibition of ACE and specific blockade of AT1R have been the major therapeutic strategies for the treatment of hypertension and other cardiovascular diseases (Cushman and Ondetti 1981; Eberhardt *et al.* 1993). However, the idea has been challenged in the last few years, with many components such as (pro)renin receptor (Nguyen and Danser 2008), Ang-(1–7) (Santos *et al.* 2005; Ocaranza *et al.* 2006), ACE2 (Lazar-

tigues *et al.* 2007) and the G protein-coupled receptor Mas (Santos *et al.* 2003) added to the classical RAS and others like Ang-(1-12) (Trask *et al.* 2008), ACE3 (Rella *et al.* 2007), Ang-A (Jankowski *et al.* 2007) and the non-AT1 non-AT2 binding site (Karamyan and Speth 2008) awaiting to be recognized.

ACE2, one of the new elements of the RAS, was identified by two groups in 2000 (Donoghue *et al.* 2000; Tipnis *et al.* 2000). The human ACE2 (hACE2) protein is a typical zinc metallopeptidase comprising 805 amino acids. As a homologue of ACE, ACE2 shares 42% sequence

Table 1 Active angiotensin peptides, receptors and major responses associated to their activation in the brain

Peptide	Receptor	Major responses
Ang-II	AT1a	Increased sympathetic tone, blood pressure, water intake, salt appetite, vasopressin release and impaired cardiac baroreflex sensitivity.
	AT1b	Water intake (?)
	AT2	Vasodilatation, anti-proliferation and cerebral ischemia
	Non-AT1 non-AT2	?
Ang-(1–7)	Mas	Increased cardiac baroreflex sensitivity, prostaglandin synthesis, vasopressin release, Nitric oxide release and substance P release.
	Other (?)	?
Ang-III	AT1	Vasoconstriction, proliferation, hypertrophy
Ang-IV	AT4/IRAP	Learning and memory, increased cerebral blood flow, hypertension and NO release (?)

identity with ACE in the metalloprotease catalytic regions (Tipnis *et al.* 2000), but unlike ACE, the carboxypeptidase hydrolyses its substrates by removing a single amino acid from their respective C-terminal. ACE2 is able to cleave the decapeptide Ang-I and octapeptide Ang-II to Ang-(1–9) and Ang-(1–7), respectively (Tipnis *et al.* 2000; Vickers *et al.* 2002; Danilczyk *et al.* 2003). The affinity for Ang-I is poor in comparison with ACE, therefore, the conversion of Ang-I to Ang-(1–9) is not of physiological importance, except maybe under conditions in which ACE activity is inhibited (Guy *et al.* 2005) or Ang-I levels are increased. It has been established that ACE2 has approximately a 400-fold greater affinity for Ang-II than Ang-I (Vickers *et al.* 2002). Hence, the major role of ACE2 in Ang peptides metabolism is the production of Ang-(1–7). ACE2 also participates in the metabolism of other peptides non-related to the RAS: apelin-13, neurotensin, kinetensin, dynorphin, [des-Arg^9]-bradykinin, and [Lys-des-Arg^9]-bradykinin (Vickers *et al.* 2002). However, the implications of ACE2-mediated metabolism of these peptides have not been investigated yet.

The distribution of ACE2 has been addressed by several groups. High ACE2 gene expression was initially reported in the heart, kidney and testis (Donoghue *et al.* 2000; Tipnis *et al.* 2000). Later studies showed ACE2 expression in a wide variety of tissues, including the brain and most of the cardiovascular-relevant tissues (Igase *et al.* 2005; Sakima *et al.* 2005; Doobay *et al.* 2007), and the current consensus is that the distribution of the protein is ubiquitous.

Ang-(1–7), the main product of Ang-II degradation by ACE2, has opposite properties to Ang-II. By acting through Mas (Santos *et al.* 2003), Ang-(1–7) promotes vasodilation, antiproliferation and antihypertrophy (Santos *et al.* 2000, 2003; Ferrario *et al.* 2005a). Accumulating evidences indicate that Ang-(1–7) has beneficial effects in cardiovascular diseases. By cleaving Ang-II into Ang-(1–7), ACE2 may play a pivotal role in counter-regulating the actions of the well documented ACE/Ang-II/AT1R axis and be beneficial for the cardiovascular system.

It has been shown that ACE2 gene is localized in a hypertension-related quantitative trait locus on the X chromosome (Crackower *et al.* 2002), suggesting that ACE2 is a putative candidate gene for hypertension. In addition, several studies have shown a strong association of the ACE2 gene polymorphism to hypertension in female Chinese patients with metabolic syndrome (Zhong *et al.* 2006) or essential hypertension (Yi *et al.* 2006; Fan *et al.* 2007). Other studies demonstrated that polymorphism of the ACE2 gene is associated with left ventricular hypertrophy in patients with hypertrophic cardiomyopathy, but the association is independent of BP (Lieb *et al.* 2006; van der Merwe *et al.* 2008; Wang *et al.* 2008). Finally, an association between ACE2 polymorphisms and coronary heart disease and myocardial infarction was observed in Chinese Han population (Yang *et al.* 2006). These studies support the idea that ACE2 plays a critical role in the regulation of BP and cardiovascular function.

Here, we will review the evidence for the involvement of ACE2 in the regulation of cardiovascular function. Following a summary of the recently discovered properties of ACE2 in the periphery (i.e., in the heart, kidney and lung), we will focus on the central role of this enzyme and discuss its potential as a new target for the treatment of hypertension and other cardiovascular diseases.

ACE2 in the periphery

ACE2 is highly expressed in the heart, kidney, and vasculature (Harmer *et al.* 2002). In the kidney, it is predominantly expressed in the proximal tubular brush border (Tikellis *et al.* 2003; Hamming *et al.* 2004), where ACE is also present (Sibony *et al.* 1993). It has also been shown in distal tubules and to a much lesser extent in glomeruli (Ye *et al.* 2004; Gembardt *et al.* 2005; Li *et al.* 2005). In the heart, ACE2 is localized to the endothelial and smooth muscle cells of intra-myocardial vessels, as well as on cardiac myocytes (Donoghue *et al.* 2000; Tipnis *et al.* 2000; Burrell *et al.* 2005). The enzyme has also been found in the thoracic aorta, carotid

arteries, and veins (Igase *et al.* 2005; Sluimer *et al.* 2008). Since its discovery in 2000, accumulating evidence has indicated that ACE2 plays a significant role in the regulation of BP, renal and cardiac functions, and its altered expression is associated with major cardiac and renal pathophysiologies.

It has been hypothesized that disruption of the balance between ACE and ACE2 would result in abnormal BP control (Yagil and Yagil 2003), therefore ACE2 might protect against increases in BP and ACE2 deficiency might lead to hypertension. Indeed, patients with hypertension showed marked ACE up-regulation and ACE2 down-regulation in both heart and kidney (Wakahara *et al.* 2007; Koka *et al.* 2008). ACE2 levels have been reported in the kidneys of three hypertensive rat strains (Crackower *et al.* 2002). In salt-sensitive Sabra hypertensive (SBH/y) rats, ACE2 mRNA and protein expression are lower than that in salt-resistant Sabra normotensive (SBN/y) rats. Following salt loading, an additional increase in BP was observed in SBH/y rats, which was correlated with a further decrease in ACE2 expression. In addition, a significant decrease of ACE2 expression was also observed in the kidneys of both spontaneously hypertensive rats (SHR) and spontaneously hypertensive-stroke prone rats as compared with their Wistar-Kyoto (WKY) controls. Similar observations were made by Zhong *et al.* showing that SHR have lower cardiac and renal ACE2 mRNA and protein (Zhong *et al.* 2004). Chronic all-*trans* retinoic acid treatment increases gene and protein expression of ACE2 in both heart and kidney, resulting in the reduction of BP and the attenuation of myocardial damage in SHR (Zhong *et al.* 2004). Treatments with ACE inhibitors or AT1R blockers increased cardiac and renal ACE2 expression and/or activity and decreased BP in Lew.Tg (mRen2) congenic hypertensive rats (Jessup *et al.* 2006). Over-expression of ACE2 in vascular smooth muscle using the SM22 promoter reduced BP in spontaneously hypertensive-stroke prone rats, and attenuated the pressor response to Ang-II in these animals (Rentzsch *et al.* 2008). Altogether, these studies suggest an important role for ACE2 in the regulation of BP.

Crackower *et al.* first demonstrated that loss of ACE2 in mice results in contractile dysfunction, increased Ang-II levels and up-regulation of hypoxia-induced genes in the heart (Crackower *et al.* 2002). The cardiac phenotype was completely rescued by genetic ablation of ACE in ACE2 knockout (ACE2$^{-/y}$) mice (Crackower *et al.* 2002), suggesting that cardiac function is modulated by the balance between ACE and ACE2. Although these cardiac abnormalities failed to be detected by Yamamoto *et al.* in their own ACE2$^{-/y}$ mouse, the authors reported the development of cardiac hypertrophy and dilatation associated with reduced cardiac contractility in response to chronic pressure overload, induced by transverse aortic constriction (Yamamoto *et al.* 2006). These authors also observed increased cardiac Ang-II levels and that inhibition of the AT1R attenuated the

hypertrophic response. Recently, Oudit *et al.* showed that loss of ACE2 leads to a progressive age-dependent dilated cardiomyopathy, associated with increased Ang-II-mediated oxidative stress (Oudit *et al.* 2007). Using similarly engineered mice, Gurley *et al.* failed to see a cardiac phenotype but observed a small elevation of baseline BP in knockout C57Bl/6 mice, no changes in ACE2$^{-/y}$ with a 129/SvEv background and variable changes in mixed animals (Gurley *et al.* 2006; Gurley and Coffman 2008), suggesting that the role of ACE2 could be modulated by genetic factors. In addition, deletion of the ACE2 gene in male mice leads to Ang-II-dependent development of glomerular mesangial expansion (Oudit *et al.* 2006). Altogether, these findings suggest an important role for ACE2 in counteracting the effects of accumulating Ang-II. However, transgenic mice with ACE2 expression targeted to myocardial cells developed spontaneous episodes of ventricular tachycardia and fibrillation leading to a fatal outcome (Donoghue *et al.* 2003), suggesting that ACE2 possibly influences the electrical pathways of the heart.

Early increases and late decreases in cardiac ACE2 expression were observed in experimental myocardial infarction in rats (Ocaranza *et al.* 2006). Cardiac hypertrophy, fibrosis, and hypertension have been associated with significant increases in cardiac ACE2 gene expression and activity in rats (Burchill *et al.* 2008). Increased ACE2 was also observed in cardiac tissue of patients with ischemic heart failure (Zisman *et al.* 2003). Moreover, blocking the effects of Ang-II by ACE inhibitors or AT1R blockers increased cardiac ACE2 expression in myocardial infarcted animals (Ishiyama *et al.* 2004; Ferrario *et al.* 2005c; Karram *et al.* 2005; Ocaranza *et al.* 2006; Burchill *et al.* 2008), suggesting that ACE2 contribute to the cardio-protective effects of ACE inhibitors and AT1R antagonists. These observations may imply that up-regulation of ACE2 is a compensatory response to the ischemic and hypertrophy insult.

This concept is supported by several studies showing that cardiac over-expression of ACE2 by lentiviral delivery exerts protective effects over: Ang-II-induced cardiac hypertrophy and fibrosis in Sprague-Dawley rats (Huentelman *et al.* 2005), hypertension in SHR (Diez-Freire *et al.* 2006) and a protective influence on the heart during myocardial infarction (Der Sarkissian *et al.* 2008).

Other studies support the assumption that ACE2 provides renal protective effects. Decreased ACE2 levels in the kidney have been shown in several diabetic animal models and patients with diabetes (Tikellis *et al.* 2003; Ye *et al.* 2006; Mizuiri *et al.* 2008). Chronic blockade of ACE2 in streptozotocin-treated mice increased albuminuria and worsened glomerular injury, which was associated with enhanced ACE expression in both glomeruli and vasculature (Soler *et al.* 2007). Inhibition of ACE2 in db/db mice also increased albuminuria and glomerular deposition of fibronectin (Ye *et al.* 2006). In line with the beneficial effects of RAS

blockade on cardiac ACE2, treatment with ACE inhibitors or AT1R blockers increased renal ACE2 levels and/or activity and reduced renal damage (Tikellis *et al.* 2003; Ferrario *et al.* 2005b; Jessup *et al.* 2006).

ACE2 also plays a protective role in the vasculature. Lower ACE2 activity has been shown in stable advanced, and in ruptured, atherosclerotic lesions (Sluimer *et al.* 2008). Local over-expression of ACE2 in a rabbit model of atherosclerosis attenuated the progression of early athero-sclerotic lesions and resulted in stable plaque compositions at late stage (Dong *et al.* 2008). Systemic blockade of AT1R increases ACE2 and Ang-(1–7) levels in both thoracic aorta and carotid artery of SHR, which are associated with attenuation of hypertrophic remodeling and neointima in these areas (Igase *et al.* 2005, 2008). These observations suggest that locally generated Ang-(1–7) through increased ACE2 expression may contribute to the reversal of vascular hypertrophy.

In addition to its interaction with the cardiovascular system, ACE2 was also identified as a functional receptor for the severe acute respiratory syndrome (SARS) coronavirus (CoV) (Li *et al.* 2003), and is expressed in type I and type II alveolar epithelial cells, bronchiolar epithelial cells, endo-thelial cells and arterial smooth muscle cells of the lung (Hamming *et al.* 2004). Down-regulation of ACE2 was found in the lungs of mice after acute lung injury, including SARS-CoV infection (Kuba *et al.* 2005) and loss of ACE2 expression in mutant mice, precipitates severe acute lung failure (Imai *et al.* 2005). Treatment with recombinant ACE2 protein attenuates acute lung failure in wild-type as well as in ACE2 knockout mice (Imai *et al.* 2005), suggesting that ACE2 plays a protective role in acute lung injury.

In summary, these studies indicate that peripheral ACE2 exerts a pivotal role in BP regulation as well as in cardiovascular, renal and pulmonary function.

ACE2 in the brain

The distribution of ACE2 in the brain was at first contro-versial as original reports failed to identify the carboxy-peptidase in the CNS (Donoghue *et al.* 2000; Tipnis *et al.* 2000). Later, low levels of ACE2 mRNA were shown in the human brain using quantitative real-time RT-PCR (Harmer *et al.* 2002), while immunohistochemistry showed that ACE2 protein expression was restricted to endothelial and arterial smooth muscle cells (Hamming *et al.* 2004). In addition, studies performed in brain primary cell cultures reported that ACE2 was expressed predominantly in glial cells (Gallagher *et al.* 2006), although this observation could be dependent on the culture conditions and the difficulty of maintaining live neurons in such cultures. SARS-CoV has been detected in brains of infected patients, almost exclu-sively in neurons, suggesting the distribution of ACE2 to the CNS (Ding *et al.* 2004; Gu *et al.* 2005; Xu *et al.* 2005).

Indeed, we recently demonstrated the presence of the ACE2 protein and mRNA in the mouse brain, predominantly in neurons (Doobay *et al.* 2007). Using a selective antibody, we found that ACE2 is widespread throughout the brain, present in nuclei involved in the central regulation of cardiovascular function like the cardio-respiratory neurons of the brainstem, as well as in non-cardiovascular areas such as the motor cortex and raphe (Doobay *et al.* 2007). Our observation was later confirmed by Lin *et al.* showing the presence of ACE2 mRNA and protein in the mouse brainstem (Lin *et al.* 2008). While these findings suggest that ACE2 is a new component of the brain RAS, they also imply that the involvement of ACE2 in the CNS is beyond the regulation of cardiovascular function.

There is much information to show that a hyperactive brain RAS plays a critical role in the development and maintenance of hypertension (Buggy *et al.* 1984; Fink *et al.* 1987; Gutkind *et al.* 1988; Gyurko *et al.* 1993; Ambuhl *et al.* 1995). In normotensive models, Ang-II acting on brain AT1R (Fink *et al.* 1987; Gutkind *et al.* 1988) induces an increase in BP mediated by enhanced sympathetic outflow (Falcon *et al.* 1978; Blume *et al.* 1999), vasopressin release (Unger *et al.* 1981) and cardiac baroreflex resetting (McDon-ald *et al.* 1980) (Table 1). In SHR, up-regulation of brain RAS components (AGT, Ang-II, ACE and AT1R) precedes and sustains the development of hypertension (McDonald *et al.* 1980; Okuno *et al.* 1983; Hermann *et al.* 1984; Casto and Phillips 1986; Gutkind *et al.* 1988; Tamura *et al.* 1996). Although the precise mechanisms by which Ang-II triggers hypertension is not known, it seems to involve increased sympathetic vasomotor tone and altered cardiac baroreflex function (Chapleau and Abboud 2001).

The latest working model of the brain RAS (Fig. 1) includes ACE2, Ang-(1–7) and Mas, therefore forming a new arm for this system (Phillips and de Oliveira 2008). While the physiological role of central ACE2 is just beginning to be addressed, there is considerable evidence for a role of Ang-(1–7) in the brain. This peptide is mainly present in central nuclei related to BP regulation, such as brainstem areas and hypothalamus (Chappell *et al.* 1989), and exerts synergistic or opposite effects to Ang-II (Moriguchi *et al.* 1995; Santos *et al.* 2000; Gironacci *et al.* 2004; Becker *et al.* 2005). Ang-(1–7) has been shown to act as an important neuromodulator of cardiac baroreflex mechanisms, leading to an increased sensitivity of this system (Campagnole-Santos *et al.* 1992; Santos *et al.* 2003). In addition, central Ang-(1–7) prevents norepinephrine release (Gironacci *et al.* 2004) and induces depressor responses (Moriguchi *et al.* 1995; Dobruch *et al.* 2003; Höcht *et al.* 2008) in hypertensive rats, increases bradykinin levels (Lu *et al.* 2008), potentiates the hypo-tensive effects of bradykinin (Bomtempo *et al.* 1998) and increases vasopressin (Moriguchi *et al.* 1994) and nitric oxide (NO) release (Gironacci *et al.* 2000). These effects are mediated by Mas (Gironacci *et al.* 2004; Höcht *et al.* 2008)
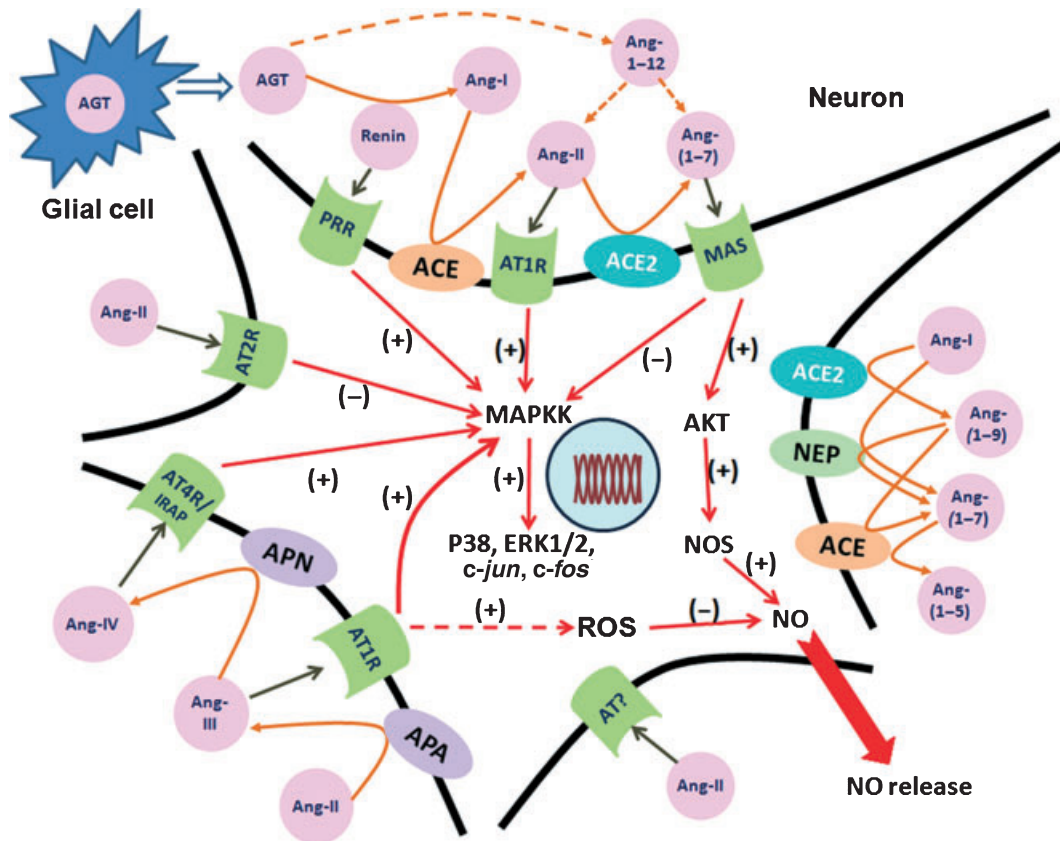
Fig. 1 Working model for the brain renin-angiotensin system. Angio-tensinogen produced by glial cells is transformed by renin to form Ang-I, which is then converted by ACE into Ang-II. This octapeptide can be hydrolyzed by ACE2 to form Ang-(1–7) or converted to the heptapepetide Ang-III by aminopeptidase A (APA) and further degraded to Ang-IV by aminopeptidase N (APN). ACE2 also cleaves Ang-I to Ang-(1–9), the latter being converted by ACE into Ang-(1–7). Ang-(1–7) can also be formed by neprilysin (NEP) from Ang-I or Ang-(1–9). ACE metabolizes Ang-(1–7) to the inactive peptide Ang-(1-5). The recently discovered Ang-(1-12) could originate from AGT and potentially generate Ang-II or Ang-(1–7). Ang-II acting on AT1 receptors (AT1R) can activate mitogen-activated protein kinase kinase (MAPKK), leading to enhanced p38 kinase activity, phosphorylation of ERK1/2 and increased expression of c-*jun* and c-*fos*. AT1R also ultimately increase reactive oxygen species (ROS) production via NAD(P)H oxidase (not represented here). On the other side AT2R oppose AT1R-mediated signaling. Ang-(1–7) acting on the Mas receptor may attenuate the actions of the ACE/Ang-II/AT1R pathway through inhibition of the MAPKK pathway and stimulation of nitric oxide (NO) release. In addition to Ang-II, renin binding to (Pro)renin receptors (PRR) and Ang-III binding to AT1R also trigger activation of the MAPKK–ERK1/2 signaling pathway. AT4R/IRAP activation induces c-*fos* expression and probable NO release.

which has been identified as the first Ang-(1–7) binding site (Santos *et al.* 2003). The brain was also the first organ in which this receptor was found to be highly expressed (Young *et al.* 1988). Moreover, Becker *et al.* recently showed the presence of Mas in cardiovascular and hydroelectrolytic control areas of the rat brain supporting the role of the Ang-(1–7) receptor in these processes (Becker *et al.* 2007).

Recent data have emerged, showing the participation of ACE2 in the brain RAS. Using SELDI-TOF Mass Spectrometry, Elased *et al.* reported that the mouse brain is the seat of high ACE2 activity while ACE activity appears to be low in the CNS (Elased *et al.* 2008). Although these data indicate that central ACE2 is active and plays a predominant role in the processing of Ang-II into Ang-(1–7) in the brain,

they are also challenging the importance of ACE in the CNS. Surprisingly, the authors suggested that central ACE2 is more important than ACE activity in normal conditions while it may be otherwise in pathological situations. This is in disagreement with previous observations showing that the physiological importance of central Ang-(1–7) is unveiled in pathological conditions and that its role is limited in normal situations (Dobruch *et al.* 2003). Moreover, in humans, the expression of ACE2 mRNA was reported to be at least 10-fold lower than ACE in the brain (Harmer *et al.* 2002). Although it is possible that mRNA levels do not correlate with ACE2 activity, validation of the SELDI-TOF Mass Spectrometry technique with current methodologies is needed. Finally, the authors' report that ACE2 activity was

restricted to the murine hypothalamus is conflicting with our own observations showing widespread distribution of this carboxypeptidase throughout the mouse CNS (Doobay *et al.* 2007) and their recent report of ACE2 expression in the brainstem (Lin *et al.* 2008).

Several studies have described the interactions between ACE2 and other components of the RAS in the periphery (Tikellis *et al.* 2003; Ferrario *et al.* 2005b; Jessup *et al.* 2006) but very few have addressed these relations in the brain. Using transgenic mice over-expressing the rat AT1a receptor selectively in the brain (NSE-AT1a mouse) (Lazartigues *et al.* 2002) or mice with chronic expression of both human renin and human AGT genes (R^+A^+ mouse) (Merrill *et al.* 1996; Davisson *et al.* 1998), we previously showed that over-expression of these central RAS components increases ACE2 protein expression in the subfornical organ (SFO) and differentially changed the expression in the brainstem (Doobay *et al.* 2007), supporting the idea that ACE2 acts as a compensatory mechanism to limit brain RAS hyperactivity. In support of this concept, Lin *et al.* using a gene silencing approach demonstrated that a reduction in AT1R mRNA is associated with a reduction in ACE2 mRNA in the brainstem (Lin *et al.* 2008). Moreover, we recently showed, for the first time, that ACE2 over-expression in the SFO resulted in the down-regulation of AT1R in this region (Feng *et al.* 2008b), suggesting that the carboxypeptidase can affect AT1R transcription and/or internalization. However, the lack of available tools to manipulate ACE2 expression has only allowed investigations of the effects of the classic RAS components on ACE2 and additional studies are needed to address the other side of the story.

As mentioned previously, ACE2 also participates in the metabolism of non-RAS peptides including Apelin-13, neurotensin and bradykinin fragments (Vickers *et al.* 2002). In the brain, Apelin-13 contributes to increase BP (Seyedabadi *et al.* 2002; Kagiyama *et al.* 2005) and seems to oppose the vasopressin antidiuretic response (Llorens-Cortes and Kordon 2008), while neurotensin (Carraway and Leeman 1973) and bradykinin produce hypotensive effects. However, despite the identification of these peptides as ACE2 substrates, there has not been any study addressing the impact of the enzyme on these neuromediators or, vice versa, the role of these peptides in the actions of ACE2 in the brain.

From gene deletion to gene therapy

Among the three ACE2$^{-/y}$ models generated, one showed a modest but significant increase in BP that appeared to be dependent upon the C57Bl/6 genetic background (Gurley *et al.* 2006). These original observations led us to reconsider the role of endogenous ACE2 in the central regulation of BP. Using the same ACE2$^{-/y}$ mouse model, we investigated spontaneous baroreceptor reflex and autonomic function. We observed that ACE2 gene deletion resulted in impaired

resting baroreflex sensitivity (Whitaker *et al.* 2007), increased sympathetic tone and decreased parasympathetic tone (H. Xia and E. Lazartigues, unpublished data). Interestingly, these observations are consistent with subjects presenting a hyperactive RAS like in hypertension and heart failure models (Zucker 2006; Grassi *et al.* 2007). However, measurement of Ang-II levels in the plasma and the brain of these mice showed no differences between wild-type and ACE2$^{-/y}$ genotypes, suggesting that alteration of baroreflex and autonomic function are not triggered by increased Ang-II levels in the CNS or at the periphery. Previous studies in the periphery have shown the beneficial effects of AT1R blockers in increasing ACE2 mRNA, suggesting that AT1R inhibit ACE2 expression (Ferrario *et al.* 2005c). In addition, our recent observation that ACE2 over-expression in the brain results in reduced AT1R levels (Feng *et al.* 2008b), implies that ACE2 is also capable of modulating the expression level of RAS components. Therefore, it is conceivable that ACE2 deletion would result in AT1R up-regulation in the brain of ACE2$^{-/y}$ mice which could then account for the impaired autonomic and baroreflex function in this model. In support of this hypothesis, preliminary data show that central blockade of AT1R by intra-cerebroventricular administration of losartan restored baroreflex sensitivity and decreased BP in ACE2 null mice but not in wild-types (Whitaker *et al.* 2007; Xia *et al.*, unpublished data), emphasizing the critical role of ACE2 in the central regulation of BP. Additional evidences were recently provided by Diz *et al.* showing that inhibition of ACE2 activity in the nucleus of the solitary tract attenuates baroreflex function for heart rate control in response to increases in BP (Diz *et al.* 2008).

Recently, we and others have developed viral vectors to over-express ACE2 in the brain with the ultimate goal of reducing brain RAS hyperactivity associated with cardiovascular diseases. Pioneering this type of experiments with ACE2 at the periphery, Dr. Raizada's group first showed that ACE2 expression was reduced by 40% in the rostral ventrolateral medulla (RVLM) of SHR compared with normotensive WKY rats (Yamazato *et al.* 2007). Interestingly, the authors showed that lentivirus-mediated ACE2 over-expression targeted to the RVLM resulted in the reduction of hypertension in SHR starting 4 weeks after infection. Although lentivirus-mediated gene expression can occur as early as 3 days post-infection, the delayed antihypertensive response observed in this study could be due to an insufficient degree of transgene expression. Indeed, while the lentivirus increased the carboxypeptidase expression by 45% in the RVLM compared with untreated SHR, ACE2 protein levels remained 22% lower than that of WKY rats (Yamazato *et al.* 2007). The mechanism by which ACE2 mediated the reduction of hypertension was not addressed in this study; however, because injection of Ang-(1–7) in the RVLM has previously been shown to increase BP (Silva *et al.* 1993), it is unlikely that this peptide mediated the

anti-hypertensive response in SHR. This decrease was probably resulting from the degradation of Ang-II by ACE2 and/or down-regulation of AT1R in this brain region. This interpretation is consistent with our own work, using an adenovirus coding for ACE2 (Feng *et al.* 2008b). While we showed that ACE2 over-expression in the SFO prevented the Ang-II-mediated pressor and drinking responses, administration of an Ang-(1–7) receptor antagonist failed to restore the enhanced BP response. On the other hand, we demonstrated that adenovirus-mediated ACE2 over-expression resulted in the down-regulation of AT1R in the SFO, thus impairing Ang-II downstream signaling (Feng *et al.* 2008b). In addition, using this adenovirus, we showed that over-expression of ACE2 in the paraventricular nucleus attenuates the increased sympathetic vasomotor tone resulting from Ang-II infusion in ACE2$^{-/y}$ mice, and reduced the associated oxidative stress in the paraventricular nucleus and downstream nuclei (Xia *et al.* 2008).

Although these studies provide the first evidence for the beneficial effects of ACE2 over-expression in the CNS, long term and high level expression is needed to dissect the mechanism(s) of action and the regulation of this enzyme in the brain.

New strategies and new tools

Determination of the role of ACE2 in the CNS (and at the periphery) has been impaired by the lack of tools to manipulate its expression and mechanism of action.

Two pharmacological antagonists have been generated and reported as highly selective at inhibiting ACE2 activity, however, their use has been limited (Dales *et al.* 2002; Huang *et al.* 2003). The first one, originally known as compound 16 or C16, was generated by Millennium Pharmaceuticals and became known as MLN4760. Although capable of inhibiting ACE2 in the picomolar range and presenting excellent selectivity versus ACE and carboxypeptidase A (Dales *et al.* 2002), its non-commercial status and intellectual property rights have confined its use to a limited number of investigators. The second compound, DX600, is a peptide inhibitor, commercially available, showing mixed competitive and non-competitive inhibition of the carboxypeptidase with very high potency (Ki = 2.8 nM) (Huang *et al.* 2003). This inhibitor has been used extensively *in vitro* (Li *et al.* 2005; Elased *et al.* 2008) and although data indicate that it might be effective *in vivo*, the doses needed and the associated cost make it prohibitive for this type of experiments.

Recently, using a virtual screening technique based on the structure of ACE2, Ferreira *et al.* identified two selective ACE2 activators: Xanthenone and resorcinolnaphthalein, both capable of enhancing ACE2 activity in a dose-dependent manner with EC$_{50}$ values of 20.1 ± 0.8 and 19.5 ± 0.4 μM, respectively (Hernandez Prada *et al.* 2008).

Interestingly, these agents were also able to increase ACE2 mRNA levels by two-fold, suggesting that the beneficial effects of these compounds might result from both increased ACE2 gene transcription and modulation of ACE2 activity. While there has not been any study of the effects of these compounds on central ACE2 expression and/or activity, *in vivo* studies showed that chronic subcutaneous infusion of Xanthenone decreased BP, improved cardiac function and reversed fibrosis in SHR (Hernandez Prada *et al.* 2008). These findings suggest that the development of ACE2 activators may provide a new approach for investigating the role of ACE2 in the CNS.

Using a genetic approach, we recently developed a new transgenic mouse model (syn-hACE2) where the full open reading frame of the human ACE2 gene is under the control of a synapsin promoter (Fig. 2a), allowing the hACE2 protein to be expressed specifically in neurons. These mice are characterized by high ACE2 expression and activity, restricted to the CNS. Interestingly, syn-hACE2 transgenic mice are protected against RAS over-activity, as illustrated by the absence of neurogenic hypertension following chronic infusion of a subpressor dose of Ang-II (Feng *et al.* 2008a). This anti-hypertensive response was prevented by co-infusion of an Ang-(1–7) receptor inhibitor, and associated with increased NO synthases expression throughout the brain (Y. Feng and E. Lazartigues, unpublished data). These observations suggest that, although impaired Ang-II signaling may participate to the reduced high BP level in syn-hACE2 mice, Ang-(1–7) is the major mediator for this response, leading to enhanced NO synthase expression which would favor NO release, thus reinforcing autonomic and baroreflex functions towards the prevention of neurogenic hypertension in this model. In support to the alteration of Ang-II signaling in syn-hACE2 mice, is the blunted drinking response in this model following Ang-II infusion. As demonstrated early on by Fitzsimons (Fitzsimons 1971), Ang-(1–7) has no effect on water intake and this could be confirmed in syn-hACE2 by the inability of the Ang-(1–7) blocker to restore the high drinking behavior observed in non-transgenic littermates (Feng *et al.*, unpublished data). Therefore, syn-hACE2 transgenic mice constitute a new interesting model to elucidate the participation of central ACE2 in various physiological responses originating in the brain and associated to a hyperactive RAS. It is likely that this model will be very useful to assess the benefits of central ACE2 in cardiovascular pathologies such as heart failure and stroke, but also in neurological affections like Alzheimer's disease, depression, stress and impaired cognition (Phillips and de Oliveira 2008).

Over the last two decades, the characterization of the responses mediated by the brain RAS has been challenging due to the difficulty in separating central versus peripheral RAS. The use of transgenic animals with brain-targeted expression of the classic RAS components has proven
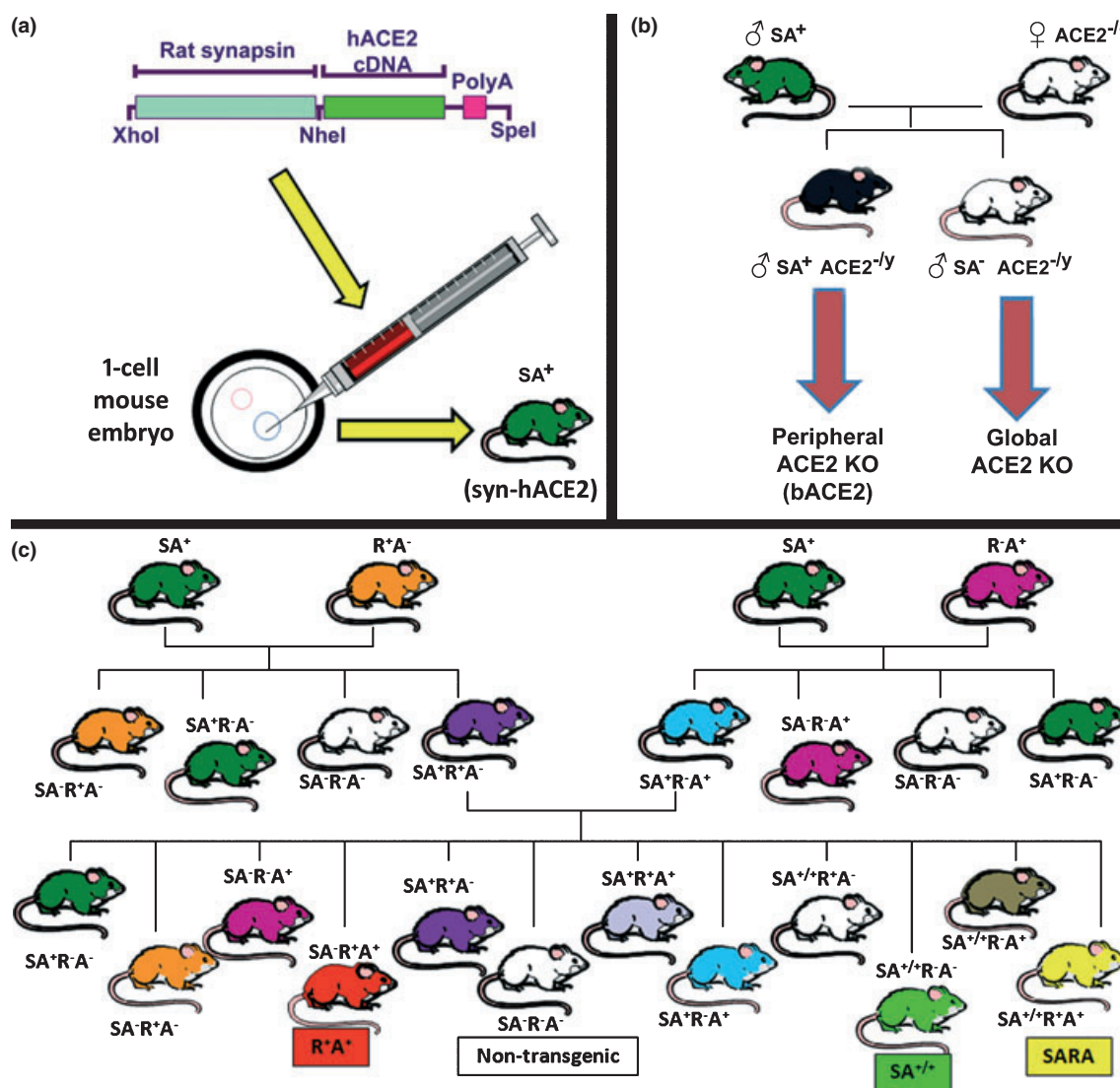
Fig. 2 Engineering of brain-selective ACE2 transgenic mouse models. (a) Syn-hACE2 mice. The full open reading frame of the human ACE2 gene (hACE2) is driven by a synapsin promoter, and the syn-hACE2 construct was injected into a 1-cell mouse embryo. Transgenic syn-hACE2 mice express hACE2 protein specifically in neurons. (b) 'Brain-only ACE2' mice (bACE2). By breeding ACE2$^{-/y}$ (white) with the syn-hACE2 line (green), we generated a transgenic mouse model over-expressing ACE2 in the brain while lacking the enzyme in the periphery (black). (c) SARA mice. The breeding strategy first consisted in generating double transgenic mice expressing both syn-hACE2 (green) and R$^+$ (human renin gene), or A$^+$ (human AGT gene) constructs. These mice were then bred together to generate the triple transgenic SARA mice (yellow) in which brain-selective over-expression of ACE2 is in a position to counter the hyperactive RAS.

extremely useful towards solving this issue (Davisson 2003; Lavoie and Sigmund 2003). ACE2 is no exception, as it is present both in the brain and the periphery. As a first step to separate central and systemic effects of ACE2, we have developed a new transgenic mouse model (bACE2) expressing ACE2 only in the brain and therefore removing the responses associated with activation of peripheral ACE2. Instead of using CRE/LoxP or shRNA systems that would require the generation and characterization of new transgenic mice, we opted for a simpler and faster strategy by taking advantage of already existing models. Using genetic complementation, our approach has been to introduce brain-selective expression of the enzyme into an ACE2 nullified background. Accordingly, by breeding ACE2$^{-/y}$ mice, lacking the ACE2 gene, with syn-hACE2 mice, expressing the hACE2 gene specifically in the CNS, we were able to generate a new mouse model with 'brain only' expression of ACE2 (Fig. 2b). Consistent with the parent strains, preliminary data indicate that resting hemodynamic parameters are normal in bACE2 mice. This new model should be helpful in addressing the participation of peripheral ACE2 in normal and pathophysiologic conditions.

Finally, in order to investigate the effects of long-term ACE2 expression on chronic hypertension, we have generated a triple transgenic mouse model (SARA mice, Fig. 2c) by transposing the syn-hACE2 phenotype onto the R^+A^+ chronically hypertensive mouse background (kind gift from Dr. Curt D. Sigmund, University of Iowa). Because of the species-specificity of the AGT and renin components, both human genes have to be present for the R^+A^+ mouse to be hypertensive (Merrill *et al.* 1996). These mice develop hypertension early on and are characterized by elevated plasma renin activity and elevated Ang-II levels. In addition, chronic over-production of Ang-II has been associated with resetting of the cardiac baroreflex (Merrill *et al.* 1996) and hemorrhagic stroke (Iida *et al.* 2005; Wakisaka *et al.* 2008) in this model. We recently observed that baroreflex sensitivity was also reduced in R^+A^+ mice while ACE2 activity was elevated in the brain (Xia *et al.* 2007). Therefore, the newly generated SARA mice constitute a new tool to investigate the ability of central ACE2 in preventing the development of chronic hypertension. In addition, they could represent an interesting model to assess the potential of brain ACE2 in limiting the occurrence of hemorrhagic stroke.

We believe that together, these models will help us to further address the contribution of central ACE2 in buffering the actions of brain and systemic Ang-II on cardiovascular function as well as neurological diseases associated with brain RAS hyperactivity.

In summary, our review of the literature shows that the long-debated physiological relevance of Ang-(1–7) is now substantiated by the identification of an enzyme leading to its formation and its own binding site, all contributing to the formation of a new axis for the RAS. Evidence is accumulating for the importance of ACE2 as a regulator of this system. Through its ability to degrade Ang-II to Ang-(1–7), ACE2 is able to regulate local Ang-II levels thereby modulating its effects. It is reasonable to conclude from the above discussion that ACE2 may play a protective role in the brain by balancing the ACE/Ang-II/AT1R axis. Disturbance of the balance could alter the levels of Ang-II and contribute to the development of hypertension and other cardiovascular diseases. The new ACE2/Ang-(1–7)/Mas axis is present in central areas related to the control of cardiovascular function and therefore provides new possibilities to counter-regulate the effects of Ang-II in the brain, and to develop new strategies for the treatment of cardiovascular and neurological diseases.

Perspectives

The discovery of ACE2 marked an important step in our vision of the RAS and a significant amount of investigators has recently joined the ranks of the 'believers', as illustrated by recent national and international conferences focusing on this aspect of Angiotensin research. However, the enlight-ened will have the difficult mission to answer outstanding questions in order to convince the other camp. Among these questions are: (i) the importance of ACE2 versus neprilysin in the formation of Ang-(1–7); (ii) the physiological relevance of Ang-I as a substrate for ACE2 *in vivo*; (iii) the ability of ACE2 to directly activate signaling pathways, as it has been proposed for ACE; (iv) the effects of ACE2 on other systems (e.g., bradykinin and opioids); and maybe the more important question: (v) the physiological relevance of ACE2 in 'normal' conditions.

As new pharmacological and genetic tools are becoming available, it is likely that future therapies will be designed to target ACE2 and will affect the treatment of diseases beyond the cardiovascular system.

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