Published in final edited form as:

Am J Physiol Regul Integr Comp Physiol. 2007 January ; 292(1): R373–R381.

Differential Expression of Neuronal ACE2 in Transgenic Mice with Overexpression of the Brain Renin-angiotensin System

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SUMMARY

ACE2 is a newly discovered carboxy-peptidase responsible for the formation of vasodilatory peptides such as angiotensin-(1-7). We hypothesized that ACE2 is part of the brain renin-angiotensin system (RAS) and its expression regulated by the other elements of this system. ACE2 immuno-staining was performed in transgenic mouse brain sections from NSE-AT_{1A} (overexpressing AT_{1A} receptors), R⁺A⁺ (overexpressing angiotensinogen, and renin) and control (non transgenic littermates) mice. Results show that ACE2 staining is widely distributed throughout the brain. Using cell type-specific antibodies, we observed that ACE2 staining is present in the cytoplasm of neuronal cell bodies but not in glial cells. In the subfornical organ, an area lacking the blood brain barrier and sensitive to blood borne angiotensin-II, ACE2 was significantly increased in transgenic mice. Interestingly, ACE2 mRNA and protein expression were inversely correlated in the nucleus of tractus solitarius/dorsal motor nucleus of the vagus and the ventrolateral medulla, when comparing transgenic to non-transgenic mice. These results suggest that ACE2 is localized to the cytoplasm of neuronal cells in the brain, and that ACE2 levels appear highly regulated by other components of the RAS, confirming its involvement in this system. Moreover, ACE2 expression in brain structures involved in the control of cardiovascular function suggests that the carboxypeptidase may have a role in the central regulation of blood pressure and diseases involving the autonomic nervous system such as hypertension.

Keywords

central nervous system; circumventricular organs; volume homeostasis; blood pressure; carboxypeptidase

INTRODUCTION

The classical view of the brain renin-angiotensin system (RAS) is an enzymatic cascade by which angiotensinogen (AGT) is successively cleaved by renin and angiotensin converting

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enzyme (ACE) then degraded by angiotensinases to form active and inactive metabolites (3, 35,40). The effects of central Ang-II, i.e. vasoconstriction, vasopressin release, induction of transcription factors, salt appetite and drinking response, are thought to be mediated primarily by AT₁ receptors (32,41). In rodents, AT₁ receptors are subdivided in AT_{1A} and AT_{1B} receptors and both are expressed in the brain (1). High density of the AT_{1A} receptor is distributed in circumventricular organs (CVOs), which lack a blood-brain-barrier (BBB) and include the subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT) and area postrema (AP) (28). Other cardiovascular (CV) regulatory areas containing AT_{1A} receptors include the median preoptic area (MnPO), paraventricular nucleus (PVN), nucleus of the tractus solitarius (NTS) and ventrolateral medulla (VLM) (32).

Recently, a new member of the ACE family was identified and named ACE2 (11,38). This carboxypeptidase was first sequenced and cloned from human heart failure (HF) ventricle (11) and human lymphoma (38) cDNA libraries. These studies reported major expression of ACE2 mRNA in heart and kidneys but failed to detect it in the brain. Interestingly, these studies also failed to detect ACE in the brain despite overwhelming reports showing its presence (28). In addition, very recent studies reported ACE2 mRNA in rat medulla oblongata (34) and ACE2 activity in mouse brain (12). ACE2 is believed to degrade Ang-II to the vasodilatory peptide angiotensin-(1-7) (Ang₁₋₇) with an affinity 400-fold greater than for Ang-I (13,42). Ang₁₋₇ is also present in the brain, where it exerts synergistic or opposite effects to Ang-II, but its receptor is still uncertain. One of the possible candidates is the G protein-coupled receptor Mas (36).

Although the presence and therefore the role of ACE2 in the brain is unknown, there is considerable evidence for a role of Ang₁₋₇. In addition to its hypotensive effects in hypertensive (5) but not in normotensive (6) rats, studies have shown that Ang₁₋₇ may act as an important neuromodulator of cardiac baroreflex mechanisms, following central (6) or peripheral (5) administration, leading to an increase sensitivity of this system (2,35). On the other hand, Ang₁₋₇ antagonists impair baroreflex sensitivity, opposing the effects of losartan on this system (31). As a new component of the RAS, controlling the production of the vasodilatory and anti-hypertrophic peptide Ang₁₋₇, ACE2 provides new possibilities to counter-regulate the effects of Ang-II and to improve the treatment of hypertension and other CV diseases. However, the relative contribution of ACE2 in the brain of normotensive and hypertensive mouse strains, in regulating blood pressure (BP) and CV function is unknown.

Here, we hypothesized that ACE2 is a component of the brain RAS and its expression is regulated by the other elements of this system. To assess this hypothesis, we used immuno-histochemistry and real-time PCR to determine the presence of the ACE2 protein and mRNA in the mouse brain as well as its cellular and regional distribution. In addition, taking advantage of NSE-AT_{1A} [with brain-selective overexpression of the rat Ang-II type 1a (AT_{1A}) receptor] and R+A+ [with widespread expression of both human renin (hRen) and angiotensinogen (hAGT) genes] transgenic mouse models exhibiting alterations of the brain RAS components, we investigated the effects of such elements on ACE2 expression in specific brain regions.

EXPERIMENTAL PROCEDURES

Transgenic mice and animal husbandry

All transgenic mice used in this study exhibit a C57BL/6J genetic background, therefore C57BL/6J non-transgenic littermates were used as controls. Transgenic mice (R⁺A⁺) harboring both hRen, (R⁺) and hAGT (A⁺) transgenes were generated by breeding heterozygous R⁺ transgenic mice with heterozygous A⁺ transgenic mice, as described previously (29,37). In this strain, the major phenotype is characterized by a chronic hypertension concomitant with high Ang-II plasma levels. Heterozygous NSE-AT_{1A} transgenic mice were generated as described

previously (24). In this strain, the rat AT_{1A} cDNA is driven by a neuron-specific enolase promoter, making its expression specific for neurons. Although not hypertensive, these mice exhibit enhanced pressor and bradycardic responses to Ang-II as well as increased baseline water intake and salt appetite. ACE2^{-/-} hemizygotes, nullified for the ACE2 gene and generated through backcrossing for 2-4 generations onto a C57BL/6J genetic background, were used for our experiments. These transgenic mice were kindly provided by Dr. Curt D. Sigmund from The University of Iowa (R⁺A⁺), Dr. Robin L. Davisson from The University of Iowa (NSE-AT_{1A}) and Drs. Susan B. Gurley and Thomas M. Coffman from Duke University (ACE2^{-/-}). All mice were fed standard mouse chow (LM-485; Teklad Premier Laboratory Diets) and water *ad libitum*. All procedures were approved by the University Animal Care and Use Committee at the University of Iowa.

Immunohistochemistry

Mice (n=5 per group) were anesthetized and perfused transcardially with 4% paraformaldehyde in phosphate buffer (PB) as described previously (24). Brains were sectioned on a cryostat and sections collected in PB. Free-floating sections (30 µm, coronal) from transgenic and non-transgenic lines were incubated in 10% normal goat serum (Sigma, St Louis, MO) for 1 h and then in 0.5% Blocking Reagent (NEN Life Science Products) for 30 min at room temperature. Sections were then incubated at 4°C with the primary antibody; a rabbit anti-mouse ACE2 antibody (kind gift of Dr. C.M. Ferrario, Wake Forest University, USA -1:5000 dilution, 48 h; see (14) for previous characterization of the antibody). This was followed by tyramide signal amplification using the ABC Vectastain® Systems kit (Vector Laboratories, Burlingame, CA), as described by the manufacturer. Briefly, sections were incubated with biotinylated goat anti-rabbit IgG (1:200,) and Streptavidin-HRP (1:100) for 1 h each. This was followed by a 10-minute amplification of biotinyl tyramide (1:100) and 1 h incubation in Rhodamine-Avidin D (1:100). In order to assess the specificity of the ACE2 staining, control sections were incubated without primary or secondary antibody. In addition, sections from ACE2^{-/-} mice were similarly processed.

Finally, sections were incubated overnight with a mouse anti-MAP-2 monoclonal antibody (1:500, Sigma) or a mouse anti-GFAP monoclonal antibody (1:500, Sigma), followed by 2 h in fluorescein-conjugated goat anti-mouse antibody (1:200, Sigma). At the end of the protocol, sections were incubated for 10 min in DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) staining for double-stranded DNA visualization. Sections were then mounted with buffered glycerol (Molecular Probes, Eugene, OR). Immunostaining was analyzed using fluorescence and UV microscopy (Zeiss LSM 510). Sections were scanned using an Argon laser emitting light at 488 nm for visualization of fluorescein and 570 nm for rhodamine.

Quantification was performed by 2 investigators using NIH Image J software, version 1.33 (<http://rsb.info.nih.gov/ij/>). The RGB confocal images were loaded into the program and converted to 8-bit gray-scale before subtracting background fluorescence equivalently for all images (setting the threshold to 50% maximum intensity). First, the image size was set by entering the appropriate dimensions using a graticule. This was followed by calibration of the optical density (OD) using a Kodak photographic step tablet. The region of interest was then outlined on the gray-scale pictures and the OD measured in this area. Fluorescence intensity is expressed as OD per square micrometer.

Real-Time Pcr

Brain tissue punches were collected from transgenic and non-transgenic mice (n=4 per group). RNA was extracted from the nucleus tractus solitarii/dorsal motor nucleus of the vagus (NTS/DMNX) and the ventrolateral medulla (VLM) using an RNeasy mini kit (Qiagen). All samples (0.5 µg) were visualized on a 1% agarose gel, in order to verify their quality. This was followed

by first-strand cDNA synthesis, from 1 µg of RNA, using SuperScript™ II RT according to the manufacturer instructions (Invitrogen). The Real-Time PCR reaction was then performed in quadruplicate using 50 ng of cDNA, mouse ACE2 primers (10 µM)(forward: 5'-ACC CTT CTT ACA TCA GCC CTA CTG-3'; reverse: 5'-TGT CCA AAA CCT ACC CCA CAT AT-3'), mouse-actin as internal control (10 µM)(forward: 5'-CCA CCA GTT CGC CAT GGA TGA-3'; reverse: 5'-ACC ATC ACA CCC TGG TGC CTA-3') (Integrated DNA Technologies, IA), and 12.5 µL of SYBR® Green PCR master mix (Applied Biosystems). The reaction mixture was placed into 1 well of a 96-well plate (Applied Biosystems), and the total reaction volume was brought to 25 µL with DEPC-treated water. PCR was performed at 50°C for 2 minutes and 95°C for 10 minutes and was run for 40 cycles at 95°C for 15 seconds and 61°C for 1 minute in an ABI Prism 7700 Detection System (Applied Biosystems). The cycle threshold for PCR amplification needed to detect fluorescence (Ct) was then determined for each unknown cDNA sample. ACE2 mRNA levels in tissues were quantified by comparison to a standard curve previously constructed for each primer set, and message levels were normalized to β -actin levels in each experiment. The Real-Time PCR reaction was performed at the University of Iowa DNA Core Facility.

Statistics

Data are expressed as mean $\pm$ SEM. Data were analyzed by one-way ANOVA (following Bartlett's test of homogeneity of variance) followed by Newman-Keuls correction for multiple comparisons between means or a Dunnett's multiple comparison test when appropriate. Statistical comparisons were performed using Prism® (version 3.0) software package (GraphPad Software Inc., San Diego, CA).

RESULTS

Cellular and regional distribution of ACE2 expression in mouse brain

The first goal of this study was to determine whether ACE2 was expressed in the brain of mice with normal expression of RAS components. Consequently, ACE2 expression was first investigated in coronal brain sections from C57BL/6J mice (non transgenic littermates). Figure 1 shows typical examples of basal ACE2 expression in the piriform cortex (Fig. 1A), caudate putamen (Fig. 1B), hypoglossal nucleus (Fig. 1C) and primary motor cortex (Fig. 1D) of C57BL/6J mice. Using similar pictures taken throughout the whole brain, assessment of ACE2 expression level was performed using a grading scale ranging from not detectable to abundant immunostaining (Table 1, Fig. 2). This approach revealed that ACE2 immunostaining is in fact widespread throughout the mouse brain, from the telencephalon to the medulla. In order to determine the type of brain cells expressing ACE2, double-immunohistochemistry was performed with the ACE2 antibody in combination with cell-specific antibodies such as GFAP (glial cell marker) and MAP2 (neuronal marker). As illustrated on Figure 1A, GFAP staining (green) in the piriform cortex does not overlap with ACE2 (red) suggesting that glial cells do not express the carboxypeptidase. Indeed, observation at a higher magnification (Fig. 1B) reveals that the size and morphology of the ACE2 expressing cells are inconsistent with glial cells and are more likely to be neuronal cells. This was confirmed by the clear overlap of MAP2 (green) and ACE2 (red) immunostainings in the hypoglossal nucleus (Fig. 1C) and primary motor cortex (Fig. 1D). Finally, ACE2 expression appears to be located in the neuron cytoplasm (Fig. 1B).

Specificity of the ACE2 antibody

The ACE2 antibody recognizes the amino-acids 198 to 216 of the mouse ACE2 protein (Sequence: DYGDYWRGDYEAEGADGYN). To verify that the ACE2 antibody was indeed targeting the ACE2 amino acid sequence, immunostaining was performed in parallel in C57BL/6J and ACE2 null (ACE2^{-/-}) mice brain sections. Figure 3 shows typical examples of ACE2

immuno reactivity in the SFO and the RVLM, respectively, of C57BL/6J and ACE2^{-/-} mice. Although the C57BL/6J mouse brain regions exhibit a fair amount of ACE2 immunostaining (3A and 3B), ACE2^{-/-} mice show no significant staining (3C and 3D), confirming the specificity of the ACE2 antibody. To confirm this result, several control experiments were also performed. Brain sections incubated with only the primary (ACE2; data not shown) or the secondary (Rhodamine-Avidin D; Fig. 3E and 3F) antibody failed to show any significant level of fluorescence, eliminating the possibility of auto-fluorescence from the brain tissue as well as other forms of nonspecific immuno-reactivity.

ACE2 protein expression in brain nuclei related to CV function

Observation of the ACE2 immunoreactivity throughout the brain of C57BL/6J mice revealed a widespread distribution, apparently independent of the involvement of any particular nuclei in CV regulation. However, according to Table 1, those CV regulatory areas (e.g. NTS, nucleus ambiguus (NA), area postrema (AP)) are more likely to express a higher level of ACE2 staining than non-CV-related regions (e.g. hippocampus). Figure 4 shows that ACE2 immunostaining was positively identified on neurons in the OVLT (Fig. 4A), SFO (Fig. 4B), PVN (Fig. 4C), AP, DMNX (Fig. 4D), NTS (Fig. 4E), NA and RVLM (Fig. 4F), suggesting that ACE2 could potentially participate in the regulation of CV function and volume homeostasis in those nuclei.

Differential ACE2 expression in transgenic mice

In order to determine whether ACE2 is an active part of the brain RAS, its expression was studied in brain tissue from mice genetically modified to overexpress components of the RAS in the central nervous system. Table 1 shows the distribution and intensity of ACE2 immunoreactivity in NSE-AT_{1A} and R⁺A⁺ mice relative to control C57BL/6J mice. Several areas, at different levels of the brain, such as the bed nucleus of the stria terminalis, the suprachiasmatic nucleus, the latero-anterior hypothalamic nucleus and the SFO revealed an increase in ACE2 immunostaining in transgenic mice compared to non transgenic controls. Figure 5A-C shows a typical example of the increase in ACE2 immunoreactivity in the SFO. Quantification of ACE2 fluorescence in this area (Fig. 5D) reveals a significant increase in NSE-AT_{1A} (0.95 ± 0.08 OD/ μm^2 , $P < 0.01$) and even greater in R⁺A⁺ (1.27 ± 0.05 OD/ μm^2 , $P < 0.001$) compared to C57BL/6J (0.54 ± 0.10 OD/ μm^2) mice. On the other hand, some areas, like the PVN (Table 1) were not significantly affected by the overexpression of brain RAS elements (C57BL/6J: 1.26 ± 0.09 , NSE-AT_{1A}: 1.27 ± 0.07 and R⁺A⁺: 1.33 ± 0.03 OD/ μm^2), while others such as the NA (Table 1) and the AP (C57BL/6J: 1.34 ± 0.12 , NSE-AT_{1A}: 1.39 ± 0.06 and R⁺A⁺: 1.11 ± 0.04 OD/ μm^2 ; $P < 0.01$) exhibited a decrease in ACE2 fluorescence in transgenic mice.

Because of the importance of brainstem nuclei in the regulation of cardiovascular function, ACE2 mRNA was quantified in parallel with the protein expression in the NTS/DMNX and the VLM. Interestingly, while the ACE2/-actin mRNA ratio was significantly increased in the NTS/DMNX of R⁺A⁺ mice (1.8 ± 0.1) compared to controls (1.0 ± 0.1 , $P < 0.05$, Fig. 6A), the protein expression was lowered (1.29 ± 0.09 vs. 1.68 ± 0.05 OD/ μm^2 , respectively, $P < 0.05$, Fig. 6C). Similarly, in the VLM, the ACE2/-actin mRNA ratio was significantly decreased in transgenic mice (C57BL/6J: 1.0 ± 0.1 ; NSE-AT_{1A}: 0.7 ± 0.1 ; R⁺A⁺: 0.5 ± 0.2 , $P < 0.05$, Fig. 6B) while protein expression increased (C57BL/6J: 1.07 ± 0.03 ; NSE-AT_{1A}: 1.32 ± 0.10 ; R⁺A⁺: 1.58 ± 0.05 OD/ μm^2 , $P < 0.05$, Fig. 6D). Taken together these observations suggest that ACE2 is highly regulated by the other components of the brain RAS.

DISCUSSION

ACE2, a single amino-acid carboxypeptidase, has recently been identified as a new member of the RAS (11,38) and is believed to hydrolyze several peptides, including Ang-II, but not

bradykinin. Early reports suggest that ACE2 may play a crucial role in CV diseases such as hypertension, myocardial infarction and heart failure by regulating the levels of Ang-II (9, 13). Despite the importance of the brain RAS in the pathogenesis of such diseases (10,46), the presence of ACE2 in the central nervous system is poorly documented. The aim of this study therefore, was to investigate ACE2 distribution and cellular expression in the brain. The major novel findings of this study are that the ACE2 protein is expressed in the brain, predominantly by neurons in areas involved in the central regulation of CV function as well as in non-CV regions. Additionally, using transgenic mice harboring alterations of the brain RAS components, we determined that ACE2 expression is regulated by the other elements of this system.

Expression of ACE2 was originally identified in the heart, kidney and testis (11,38). Later, the carboxypeptidase was also discovered to be a receptor for a coronavirus responsible for the severe acute respiratory syndrome (SARS) (27), leading to increased interest in the enzyme distribution. Using immuno-histochemistry, the list of tissue harboring ACE2 was extended to lungs, nasopharynx, skin, lymph nodes, thymus, bone marrow, spleen, liver, and gastro-intestinal tract (17). Despite recent identification of ACE2 activity (12) and mRNA (34) in rodent brain, the presence of the ACE2 protein in the brain remained uncertain.

Our study shows, for the first time, that ACE2 protein and mRNA are expressed in selective nuclei of the mouse brain. Using specific antibodies for neuronal and glial cell markers, in addition to morphological observations, we found that the cells harboring ACE2 are predominantly neurons. This finding contrast with previous studies relative to ACE2 distribution. Indeed, original reports failed to identify ACE2 in the brain (11,38). Later, quantitative real-time PCR data reported low levels of ACE2 mRNA in the central nervous system (18) while immunostainings showed that its presence was restricted to endothelial and vascular smooth muscle cells (17). Finally, studies performed in brain primary cell cultures reported that ACE2 was expressed predominantly in glial cells (15) but this observation could be dependent on the culture conditions and the difficulty in maintaining neurons in such cultures. In support of our data, real-time PCR and in situ hybridization studies showed that SARS coronavirus mRNA and virus protein immunoreactivity have been identified in cerebrospinal fluid (20) as well as brain neurons from infected patients (19,45) and mice (16), suggesting the localization of its receptor, ACE2, in such neurons. In addition, our data show a clear expression of ACE2 in the cytoplasm of neuronal cells. This cytoplasmic expression is in accordance with a previous report using human lung tissue and epithelia samples (17). Moreover, like ACE, ACE2 has been shown to exist as a membrane-bound and a secreted protein (14,33,39), consistent with the presence of the enzyme in the cytoplasm.

According to our data, distribution of ACE2 is widespread in the mouse brain, in areas involved or not in the regulation of CV function. More importantly, regional expression of ACE2 in the brain is consistent with the presence of other components of the RAS in the same areas, including AT₁ receptors (8,24), ACE (23), angiotensinases (4) but also Ang₁₋₇ (7), confirming ACE2 as a member of the brain RAS. Ang₁₋₇, the product of Ang-II hydrolysis by ACE2, has been shown, in the brain, to modulate the cardiac baroreflex mechanisms (5,6) in a similar way than following losartan administration, resulting in increased sensitivity of this system (2,35). Interestingly, when administered directly in baroreflex areas like the NTS and RVLM, Ang₁₋₇ induced depressor and pressor responses, respectively (35). These reverse effects are compelling when paralleled to our data, showing opposite changes in ACE2 levels in those areas, in the chronically hypertensive R⁺A⁺ mouse strain, providing additional evidence of the relationship between ACE2 and the regulation of CV function. In addition, previous studies have reported changes in baroreflex sensitivity in R⁺A⁺ mice (26,29) and it might be interesting to determine whether those changes in ACE2 expression levels are the cause or the consequence of the development of hypertension in those animals. Indeed, the elevated BP in this model

may have contributed to some of the changes observed in the NTS and the RVLM. For example, the high BP in R^+A^+ mice could have led to the reduction of ACE2 protein levels in the NTS of this strain. Clearly, more work is needed to identify how ACE2 is regulated, and/or is regulating BP, in these areas.

Another interesting area exhibiting enhanced ACE2 expression in hypertensive mice is the SFO, a circumventricular organ lacking blood brain barrier and sensitive to peripheral levels of Ang-II. This area has previously been shown to play a significant role in the central regulation of BP and volume homeostasis (22,28). Moreover, the SFO is also connected indirectly to the RVLM via the PVN and is in a position to modulate sympathetic outflow. Our observation of increased ACE2 levels in this region in R^+A^+ mice could support the idea of a compensatory mechanism promoting the hydrolysis of Ang-II and increasing forebrain levels of Ang₁₋₇. However, this hypothesis is hard to reconcile with previous studies performed in infarcted rat hearts (21) and in-vitro (14) and more work is needed to study our models in similar conditions. Also noteworthy is the increase in ACE2 levels in the SFO of NSE-AT_{1A}. Indeed, despite a lack of hypertension, the increase in neuronal AT_{1A} receptors in this model has been shown to lead to enhanced water and salt intakes (25). These observations suggest that the exogenous AT_{1A} receptor is able to activate downstream pathways responsible for those changes. Consequently, increased ACE2 protein levels in the SFO, and the lamina terminalis in general, may suggest the involvement of this carboxypeptidase in the central regulation of body fluid homeostasis.

An interesting finding in our study is that ACE2 mRNA levels are not correlated with the protein levels. Indeed, areas such as the dorsal (NTS/DMNX) and ventral (VLM) medulla show an inverse correlation between the message and the protein expression. A possible explanation for these discrepancies could be the lack of specificity of the tissue collection for mRNA extraction; the punches collected usually overlapping more than one area. In the brain this mismatch often results from localization of the mRNA in the areas containing the cell bodies and the protein on terminal fibers, although in our case, the majority of the staining is associated with the cell bodies. A more exciting hypothesis is that components of the RAS, e.g. the AT_{1A} receptor, might be able to directly or indirectly regulate ACE2 mRNA post-transcriptionally by processes such as increased stabilization, translation or degradation of the message. Similar mechanisms have previously been reported for ACE2 and other components of the RAS (30,43,44) and will be the subject of future investigations.

It is now well documented that Ang-II and Ang₁₋₇ produced and acting locally in the brain serve a crucial role in CV function. Moreover, there is considerable evidence supporting the sensitivity of the brain RAS to circulating Ang-II levels and the role of this system in the pathogenesis of both experimental and genetic hypertension (10). So far, the beneficial effects of the RAS blockade have been attributed to the inhibition of the vasoconstrictor and hypertrophic properties of Ang-II. Similarly to its suspected beneficial effects in the periphery (13), ACE2 in the brain appears to be in a position to buffer the excess Ang-II levels in nuclei involved in CV and autonomic regulation.

In summary, this study is the first to show the presence of the ACE2 protein and mRNA in the mouse brain, predominantly in neurons, in regions involved or not in the central regulation of CV function. Our data suggest that ACE2 is part of the brain RAS and highly regulated by the other components of this system. As such, ACE2 could play a major role in the central regulation of autonomic nervous system in buffering the enhanced Ang-II levels in diseases such as hypertension and myocardial infarction where this peptide has been shown to activate pathways leading to an increased sympathetic tone (10,46). Finally, the evidence of ACE2 as part of the brain RAS introduces a new level of regulation in this system and provides a new tool to fight diseases associated with an imbalance of the autonomic nervous system.

ACKNOWLEDGEMENTS

The work described herein was partly funded by The American Physiological Society Potdoctoral Fellowship in Physiological Genomics, a Beginning-Grant-In-Aid (0560007Z) from the American Heart Association Heartland Affiliate and a R21 grant from the National Institute of Neurological Disorders and Stroke (NS052479) to E. Lazartigues. The authors would like to thank Dr Curt D. Sigmund for providing the R⁺A⁺ mice, Drs Thomas M. Coffman and Susan B. Gurley for providing the ACE2-/y mice, and Drs Martin M. Cassell, Martine Dunnwald and Xinping Yue for their expertise.

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Figure 1.

ACE2 is expressed in the cytoplasm of neuronal cells. (A) Immuno-staining for ACE2 (red), GFAP (glial cell marker, green), and DAPI (nucleic acid marker, blue) in the piriform cortex shows a lack of co-localization between ACE2 and the astroglia (magnification 40X). (B) Increased magnification (63X) in the caudate putamen shows the absence of GFAP staining in ACE2-positive cells. Immuno-reactivity is localized in the cytoplasm and not in the nucleus. Immuno-staining for ACE2, MAP2 (neuronal marker, green), and DAPI in the hypoglossal nucleus (C) and the primary motor cortex (D) shows co-localization (yellow) between ACE2 and MAP2 suggesting that ACE2 is expressed mostly in neurons. All panels are from C57BL/6J mice.

Figure 2.

ACE2 quantification in mouse brain. Levels of ACE2 fluorescence were graded throughout the whole brain using the following: no detectable immunostaining (-), low-level immunostaining (+), abundant immunostaining (++), and highly abundant immunostaining (+++). All panels are from C57BL/6J mice.

Figure 3.

Specificity of the ACE2 antibody. Brain sections of the subfornical organ (SFO; A,C,E) and rostral ventrolateral medulla (RVLM; B,D,F) from C57BL/6J (A,B) and ACE2^{-/-} (C-F) mice were incubated with (A-D) and without (E,F) anti-ACE2 antibody. Note that ACE2 immunostaining is restricted to the SFO (A) and absent in the surrounding tissue. The lack of ACE2 immunostaining in ACE2^{-/-} mice (C-F) confirms the specificity of the anti-ACE2 antibody (scale bar: 100 μ m).

Figure 4.
ACE2 expression in key brain regions involved in the regulation of blood pressure and body fluid homeostasis. This panel shows examples of ACE2 (red) expression in (A) the organum vasculosum of the lamina terminalis (OVLT), an area involved in thirst and salt-appetite. Brain nuclei involved in the regulation of cardiovascular function such as (B) the subfornical organ (SFO), (C) the magnocellular neurons of the paraventricular nucleus (PVN), (D) the area postrema (AP), the dorsal motor nucleus of the vagus (DMNX), (E) the nucleus of tractus solitarius (NTS), (F) the rostromedullary lateral medulla (RVLM), and the nucleus ambiguus (NA) also showed positive staining for ACE2. The neuronal marker MAP2 is shown in green and cell nuclei are stained in blue. All panels are from C57BL/6J mice (scale bar: 100 μ m).

Figure 5.

ACE2 protein expression in the forebrain is genotype-dependent. Typical examples in the subfornical organ (SFO) of the differential expression of ACE2 in C57BL/6J (A), NSE-AT_{1A} (B) and R⁺A⁺ mice (C). Quantification of ACE2 immunoreactivity (D) shows that expression of the enzyme is modulated by overexpression of central AT_{1A} receptors in NSE-AT_{1A} mice and/or human renin and human AGT in R⁺A⁺ mice in the SFO. ***P* < 0.01 and ****P* < 0.001 versus non-transgenic mice (scale bar: 100 μm).

Figure 6.

ACE2 is differentially expressed in transgenic mouse brainstem. Quantification of ACE2 mRNA expression in the nucleus of tractus solitarius/dorsal motor nucleus of the vagus (NTS/DMNX) (A) and the ventrolateral medulla (VLM) (B) and protein immunostaining in the NTS (C) and the rostral ventrolateral medulla (RVLM) (D). Immunostaining and mRNA expression are inversely correlated in these areas and regulated by the other components of the RAS. * $P < 0.05$ versus C57BL/6J mice.

Table 1

Distribution of ACE2 immunofluorescence in non-transgenic and transgenic mouse brains. Summary of the regional distribution and level of ACE2 immunostaining in brains of NSE-AT1A, R⁺A⁺ transgenic mice and C57BL/6J non-transgenic mice. Levels of ACE2 fluorescence were defined as illustrated in Figure 3. Observations were made by 2 investigators, in 5 mice from each group, with the observers blind to genotype. Brain areas were identified according to a mouse brain atlas.

	C57BL/6J	NSE-AT _{1A}	R ⁺ A ⁺
Telencephalon:			
cortex (primary and secondary motor)	+++	+++	++
cingulum	-	+	-
caudate putamen	+++	+++	+++
anterodorsal preoptic nucleus	+	+	++
olfactory tubercle	+	++	++
lateral preoptic area	-	+	++
median preoptic area	++	++	++
substantia innominata	-	+	+
bed nucleus stria terminalis	+	++	+++
lateral septal nucleus	-	++	+++
medial septal nucleus	-	++	+++
ventral pallidum	-	+	++
interstitial nucleus post. limb ant. com.	+	++	++
lateral globus pallidus	-	+	++
amygdala	+	++	++
basal nucleus of Meynert	-	+	++
Diencephalon:			
anteroventral periventricular nucleus	++	+++	+++
ventromedial preoptic nucleus	++	+++	+++
ventral paraventricular thalamic nucleus	+	++	++
suprachiasmatic nucleus	+	++	+++
medial accessory optic tract	++	+++	+++
reticular thalamic nucleus	++	+++	++
mediodorsal thalamic nucleus	-	++	+++
reuniens thalamic nucleus	+	+	+++
magnocell. paravent. hypothal. nucleus	++	++	++
parvocell. paravent. hypothal. nucleus	++	++	++
periventricular hypothalamic nucleus	-	+	++
lateral hypothalamic area	+	+	++
lateroanterior hypothalamic nucleus	+	++	+++
arcuate hypothalamic nucleus	++	+++	+++
supraoptic nucleus	-	+	++
central medial thalamic nucleus	-	+	++
hippocampus	-	-	+
Pons-Midbrain:			
dorsal raphe nucleus	++	+++	+++
median longitudinal fasciculus	+	+	++
pontine reticular nucleus	+	++	+
median raphe nucleus	++	+++	+++
principal sensory trigeminal nucleus	+	+	+
parvicellular nucleus trigeminal nerve	-	+	+
motor root trigeminal nerve	+	+	++
periolivary nuclei	-	-	+
rostral periolivary region	-	-	+
nucleus lateral lemniscus	+	++	++
laterodorsal tegmental nucleus	++	++	+
subpeduncular tegmental nucleus	+	-	-
cerebellum	++	++	++
Medulla:			
nucleus tractus solitarius	+++	+++	++
dorsomedial spinal trigeminal nucleus	-	-	+
ventral cochlear nucleus	++	+++	+++
paratrigeminal nucleus	+	++	++
dorsal motor nucleus vagus	++	+++	+++
hypoglossal nucleus	++	++	++
nucleus of Roller	+	+	+
raphe magnus	+	+	+
raphe obscurus	+	+	+
rostromedial medulla	+	++	+++
lateral reticular nuclei	+++	+++	+++
nucleus ambiguus	+++	+	+
Circumventricular Organs:			
subfornical organ	+	++	+++
vascular organ of the lamina terminalis	++	++	++
median eminence	++	++	+

	C57BL/6J	NSE-AT _{1A}	R ⁺ A ⁺
area postrema	+++	+++	++