Impact of ACE2 Deficiency and Oxidative Stress on Cerebrovascular Function With Aging

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Background and Purpose—Angiotensin II produces oxidative stress and endothelial dysfunction in cerebral arteries, and angiotensin II type I receptors may play a role in longevity and vascular aging. Angiotensin-converting enzyme type 2 (ACE2) converts angiotensin II to angiotensin (1–7) and thus, may protect against effects of angiotensin II. We hypothesized that ACE2 deficiency increases oxidative stress and endothelial dysfunction in cerebral arteries and examined the role of ACE2 in age-related cerebrovascular dysfunction.

Methods—Endothelial function, expression of angiotensin system components, NADPH oxidase subunits, and proinflammatory cytokines were examined in cerebral arteries from adult (12 months old) and old (24 months old) ACE2 knockout (KO) and wild-type (WT) mice. The superoxide scavenger tempol was used to examine the role of oxidative stress on endothelial function.

Results—Vasodilatation to acetylcholine was impaired in adult ACE2 KO (24±6% [mean±SE]) compared with WT mice (52±7%; $P<0.05$). In old mice, vasodilatation to acetylcholine was impaired in WT mice (29±6%) and severely impaired in ACE2 KO mice (7±5%). Tempol improved endothelial function in adult and old ACE2 KO and WT mice. Aging increased mRNA for tumor necrosis factor-α in WT mice, and significantly increased mRNA levels of NAPDH oxidase 2, p47^{phox}, and Regulator of calcineurin 1 in both ACE2 KO and WT mice. mRNA levels of angiotensin system components did not change during aging.

Conclusions—ACE2 deficiency impaired endothelial function in cerebral arteries from adult mice and augmented endothelial dysfunction during aging. Oxidative stress plays a critical role in cerebrovascular dysfunction induced by ACE2 deficiency and aging. **(*Stroke*. 2012;43:3358–3363.)**

Key Words: aging ■ angiotensin-converting enzyme 2 ■ cerebral arteries ■ endothelium ■ oxidative stress

Stroke is the second most frequent cause of death from cardiovascular events in the Untied States.[1] Hypertension and endothelial dysfunction increase with age and are risk factors for stroke.[2–5] Aging is associated with endothelial dysfunction and oxidative stress in cerebral arteries.[6–8] In people without coronary artery disease, endothelial dysfunction is associated with a 4-fold increase in the risk of cerebrovascular events.[4] Cerebral endothelial dysfunction may also have a role in the pathophysiology of vascular cognitive impairment and Alzheimer disease.[9–11] Angiotensin II increases reactive oxygen species (ROS) and superoxide levels via increases in expression and activation of NADPH oxidases, a major source of superoxide anion in the vasculature.[12] Superoxide reacts with the vasodilator NO to produce peroxynitrite, resulting in decreased NO bioavailability and endothelial dysfunction.[13,14] Angiotensin II impairs endothelial function in cerebral arteries and the microcirculation.[14–16] Interestingly, genetic deletion of angiotensin II type 1 receptors (AT1Rs) markedly attenuates cerebrovascular dysfunction during aging[8]. Pharmacological modulation of angiotensin signaling in patients with stroke is associated with decreased inflammation, better functional outcome, and decreased risk for future cardiovascular events.[17]

Angiotensin-converting enzyme type 2 (ACE2), a homolog of ACE with different substrate specificity, metabolizes angiotensin II into angiotensin 1–7.[18,19] Binding of angiotensin (1–7) to the Mas receptor[20] attenuates signaling cascades activated by angiotensin II, decreases activity of NAPDH oxidase,[21] and produces vasodilatation.[20,22] Several studies suggest that ACE2 levels may be reduced with aging,[23,24] which should result in magnification of effects of angiotensin II. However, little is known about the function of ACE2 in cerebral arteries or in endothelial dysfunction during aging. In this study, we tested the hypothesis that ACE2 deficiency increases oxidative stress and vasomotor dysfunction in cerebral arteries and examined the effects of ACE2 on endothelial function in adult animals and during aging.

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Methods

Experimental Animals

Studies were performed in adult (12±0.2 months old [mean±SE]) and old (24±0.4 months old) male ACE2-deficient (knockout [KO]) and wild-type (WT) mice (n=54). The ACE2 gene is located in the X chromosome, and ACE2 KO mice and WT littermates were derived from breeding heterozygous females with WT or KO males.[25] The mice were bred onto a C57 background for 8 generations. All experimental protocols and procedures conform to the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Iowa.

Studies of Endothelial Function

Mice were euthanized with an overdose of sodium pentobarbital (150 mg/kg IP). The brain was rapidly removed and placed in ice-cold Krebs solution. The basilar artery was carefully isolated, removed, cannulated, and pressurized to 60 mm Hg in an organ bath. After an equilibration period of 30 minutes, baseline diameter was measured and contraction was examined in response to KCl (50 mmol/L). The arteries were submaximally constricted with the thromboxane A_2 analog U46619 before assessment of dilator responses. Endothelium-dependent vasodilatation was tested with acetylcholine (ACh). Endothelium-independent vasodilatation was tested with sodium nitroprusside and papaverine. To test the role of ROS, arteries were preincubated (30 minutes) with Tempol (1 mmol/L), a superoxide scavenger, before treatment with ACh.

Cerebral arteries were also used for analysis of gene expression and immunohistochemical studies. Plasma was collected for measurement of angiotensin levels. Detailed methods are available in the Supplemental Data.

Statistics

Results are expressed as mean±SEM. Statistical significance in assays of endothelial function was determined by repeated measures 2-way ANOVA on the complete data set. Then, the significance of comparisons within the adult or old data set was evaluated using Tukey post hoc test and the highest (10^{-4} mol/L) concentration of ACh. Comparison between adult and old mice was performed with ANOVA followed by Newman-Keuls multiple comparison test. Statistical significance for gene expression and peptide measurement assays was determined by 2-way ANOVA and Bonferroni post hoc test. Significant differences were identified when $P<0.05$. The analysis was performed using Prism 5 (Graphpad, La Jolla, CA) and was validated in SAS (SAS Institute Inc, Cary, NC).

Results

Expression of Components of the ACE2/Angiotensin 1–7/Mas Axis and the Renin Angiotensin System

ACE2 deficiency was confirmed in ACE2 KO mice using real-time quantitative polymerase chain reaction in samples from kidneys and brain arteries (Table; and Supplemental Table I.). ACE2 mRNA levels in cerebral arteries and kidneys were not significantly different between adult and old WT mice. ACE2 mRNA levels in brain cortex from adult and old WT mice did not change during aging (data not shown).

The presence of ACE2 protein was assessed using Western blotting and immunostaining. ACE2 expression was confirmed in kidney homogenates from WT mice but was absent in kidney homogenates from an ACE2 KO mouse (Figure 1B). ACE2 protein was not detected by Western blotting in homogenates from cerebral arteries (Figure 1B). ACE2 expression by immunofluorescence was abundantly localized in epithelium of renal tubules (Figure 1A). Weak positive staining for ACE2 was detected in sections from cerebral arteries, but it was difficult to differentiate from weak background staining in ACE2 KO mice (Figure 1A).

In cerebral arteries and kidneys, mRNA levels of the angiotensin 1–7 receptor Mas and the AT1R were similar in all groups (Table; and Supplemental Table I).

Effect of ACE2 Deficiency on Blood Pressure

Systolic blood pressure was comparable ($P>0.05$) between adult ACE2 KO (104±3) and WT mice (106±4 mm Hg). Similar results were found in old ACE2 KO (113±12) versus WT mice (109±6 mm Hg).

Vascular Function in Adult ACE2 KO and WT Mice

Baseline diameter of the basilar artery under resting conditions (before precontraction) was similar in ACE2 KO (163±8 µm) and WT mice (172±6 µm). Dilatation to ACh was reduced by ≈50% in the basilar artery from adult ACE2 KO mice (24±6%) compared with adult WT mice (52±7%; $P<0.05$). Tempol improved responses to ACh in both ACE2 KO (to 78±7%; $P<0.05$) and WT mice (87±13%; $P<0.05$) (Figure 2A). Vasodilatation to the endothelium-independent

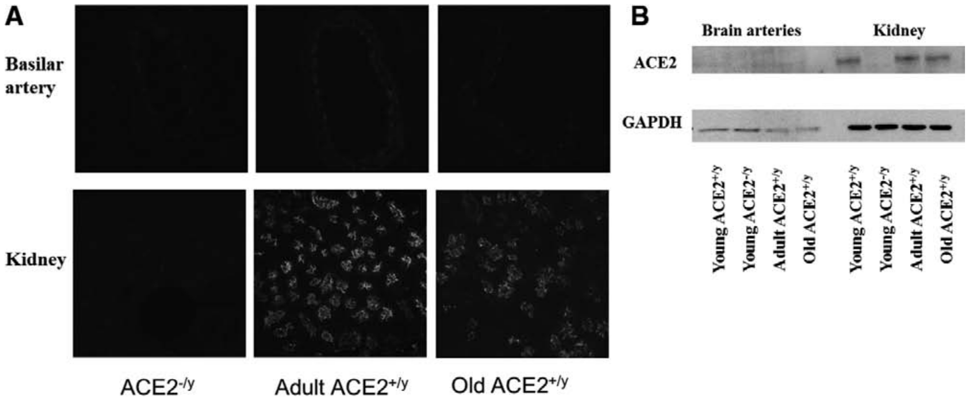

Figure 1. Angiotensin-converting enzyme type 2 (ACE2) expression in basilar arteries and kidneys. **A**, ACE2 staining in basilar artery, especially in the smooth muscle layer of adult WT mice. ACE staining was also seen in the epithelium of renal tubules in adult and old WT mice. Staining was absent in sections from ACE2 knockout (KO) mice. Similar findings were observed in 3 mice from each group. **B**, Western blots showing ACE2 expression in kidney homogenates from wild-type (WT) mice and absence of protein in the ACE2 KO mouse. ACE2 staining was not detected by Western blotting in brain arteries.

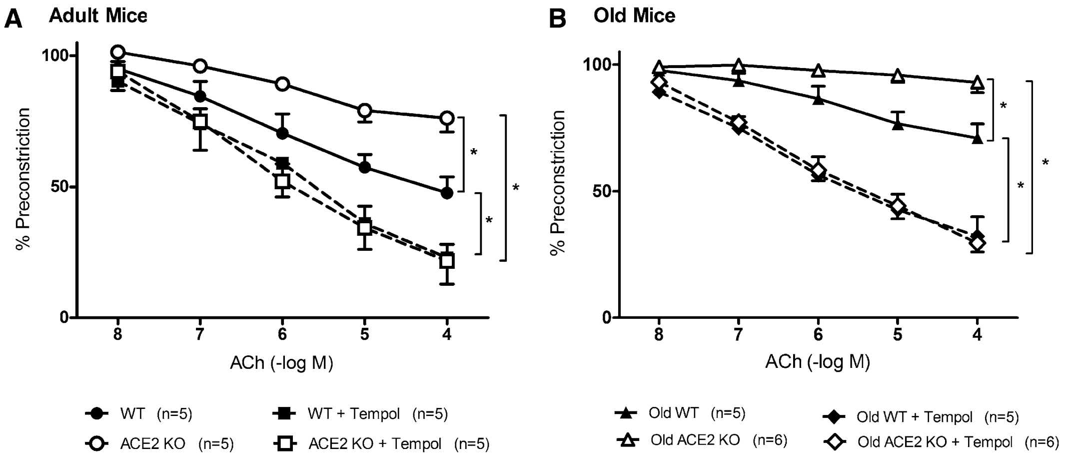

Figure 2. Effects of angiotensin-converting enzyme type 2 (ACE2) deficiency and oxidative stress on vascular function. **A**, Vasodilatation to acetylcholine (ACh) in adult ACE2 WT(●; n=5) and knockout (KO; ○; n=5) mice. Role of oxidative stress was examined after incubation with tempol of basilar arteries from adult ACE2 wild-type (WT; ■) and KO (□) mice. **B**, Vasodilatation to ACh was examined in old ACE2 WT(▲; n=5) and KO (Δ; n=6) mice. Tempol was also added to arteries from old ACE2 WT (◆) and KO (◇) mice. Values are mean ±SE; **P*<0.05.

agonists, sodium nitroprusside and papaverine was similar in both groups.

Vasoconstriction to KCl (50 mmol/L) was comparable between ACE2 KO (43±5%) and WT mice (48±3%). Vasoconstriction to U46619 was also similar in ACE2 KO (26±1%) and WT mice (25±3%).

Effect of ACE2 Deficiency and Aging in Cerebral Vascular Function

Diameter of the basilar artery was similar in old ACE2 KO (166±5 µm) versus WT (182±7 µm) mice (*P*=0.07). Maximal vasodilatation to ACh was significantly less in old WT mice than in adult WT mice (Supplemental Figure I). Similarly, maximal responses to ACh were less in old ACE2 KO mice than in adult ACE2 KO mice (*P*<0.05). In old mice, vasodilatation to ACh was profoundly impaired in ACE2 KO mice and was significantly less than in old WT mice (Figure 2B). Tempol improved responses to ACh in both old ACE2 KO (*P*<0.01) and old WT mice (*P*<0.01). Vasodilatation to sodium nitroprusside and papaverine was similar in both groups.

Vasoconstriction to KCl was also preserved in ACE2 WT (43±4%) and KO mice (51±2%). Vasoconstriction in response to U46619 was similar in old ACE2 WT (28±4%)and KO mice (32±2%).

Oxidative Stress and Inflammation

mRNA transcript levels of NADPH oxidase subunits p47^{phox} and NAPDH oxidase 2 were higher in cerebral arteries from old versus adult mice (Figure 3). Expression of the subunits was not affected by genotype (Figure 3). Nuclear factor (erythroid-derived 2)-like 2 levels were similar in the 4 groups. Expression of extracellular superoxide dismutase was increased in old WT mice (Table). Catalase was increased significantly in old ACE2 KO and WT mice (Table). Nitrotyrosine immunostaining was relatively low in sections of basilar artery from adult WT mice. Quantification of these data were difficult, but the immunostaining appeared to be increased in adult ACE2 KO mice and old ACE2 KO and WT mice (Supplemental Figure II).

Aging was associated with increased mRNA levels of tumor necrosis factor-α in WT mice. Regulator of calcineurin 1 (Rcan1) mRNA levels were also significantly increased in cerebral arteries from both ACE2 KO and WT mice. Interleukin-6 and inducible NO synthase mRNA levels were not significantly different between the groups (Table).

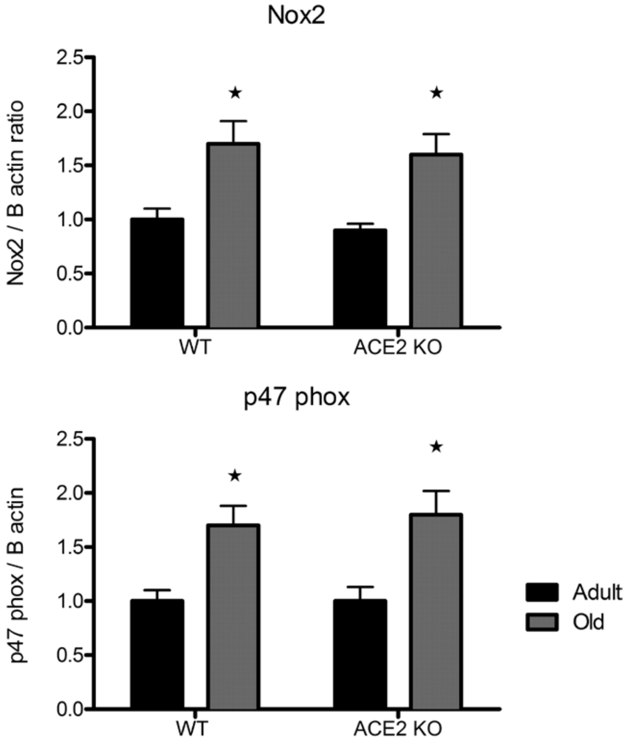

Figure 3. Effect of aging on expression of NADPH oxidase (Nox) subunits. Relative expression levels of Nox2 and p47phox mRNA in cerebral arteries from adult (**black bars**) and old (**gray bars**) wild-type (WT) or angiotensin-converting enzyme type 2 (ACE2) KO mice. Values are mean ±SE (n=7/group), **P*<0.05 vs adult mice.

Table. Gene Expression in Cerebral Arteries From Adult and Old ACE2 KO and WT Mice

	Adult		Old	
	WT	ACE2 KO	WT	ACE2 KO
ACE2	1.00 (±0.25)	0.05 (±0.03)*	1.39 (±0.17)	0.06 (±0.06)*
Mas	1.00 (±0.14)	0.86 (±0.08)	1.05 (±0.32)	0.73 (±0.05)
AT1Rs	1.00 (±0.14)	0.88 (±0.27)	1.09 (±0.14)	1.07 (±0.21)
EcSOD#	1.00 (±0.09)	1.08 (±0.11)	1.40 (±0.18)*	1.42 (±0.10)
Catalase#	1.00 (±0.09)	1.13 (±0.11)	1.44 (±0.14)*	1.78 (±0.18)†
Nrf2	1.00 (±0.05)	0.93 (±0.07)	1.11 (±0.09)	1.16 (±0.10)
IL-6	1.00 (±0.21)	0.57 (±0.21)	1.27 (±0.41)	1.76 (±0.70)
TNFα#	1.00 (±0.23)	0.99 (±0.31)	2.03 (±0.39)*	1.64 (±0.28)
Rcan1#	1.00 (±0.07)	0.98 (±0.09)	1.42 (±0.10)*	1.41 (±0.09)†
iNOS	1.00 (±0.17)	0.65 (±0.11)	1.16 (±0.24)	1.12 (±0.12)

ACE2 indicates angiotensin-converting enzyme type 2; AT1 Rs, angiotensin II type 1 receptors; EcSOD, extracellular superoxide dismutase; Nrf2, Nuclear factor (erythroid-derived 2)-like 2; IL, interleukin; TNFα, tumor necrosis factor-α; Rcan1, Regulator of calcineurin 1; iNOS; inducible NO synthase.

Data expressed as mean±SEM. n=6 to 7 mice/value.

**P*<0.05 vs adult WT.

†*P*≤0.05 vs adult KO.

#*P*<0.05 overall effect of aging (2-way ANOVA).

Levels of Angiotensins in Plasma

Angiotensin II levels were not significantly different between adult ACE2 KO and WT mice (Figure 4A). Angiotensin II levels were not significantly affected by aging although they tended to be lower in old ACE2 KO and WT mice. Likewise, angiotensin 1–7 levels were not affected by aging or genotype (Figure 4B).

Discussion

There are several major new findings in this study. First, genetic deficiency of ACE2 impaired endothelial function in the cerebral circulation. Second, cerebrovascular dysfunction during aging was greater in ACE2 KO mice than in WT mice. Third, oxidative stress plays a key role in cerebrovascular dysfunction with ACE2 deficiency and during aging. These findings provide evidence for an important role of ACE2 in the maintenance of endothelial function in cerebral arteries under normal conditions and support the overall concept that the renin angiotensin system has a major impact on the cerebrovasculature with aging. We chose to study cerebral vessels because cerebral arteries are important resistance vessels in the brain[14] and play an important role in the pathophysiology of stroke. Previous data have shown that young ACE2 KO mice have endothelial dysfunction in conduit vessels such as aorta,[26] but the effects on the cerebral circulation and mechanisms responsible for impaired vascular function in ACE2 KO mice have not been explored.

There is a poor understanding of the role of ACE2 in cardiovascular disease or stroke. Studies of ACE2 polymorphisms in patients suggest a weak association of the ACE2 G9570A polymorphism with stroke.[27] Decreased expression of ACE2 has been found in kidneys of patients with diabetes and renal disease.[28] Hypertension is an important risk factor for stroke and one might expect that ACE2 deficiency would be associated with hypertension. ACE2 deficiency, however, has little or no effect on blood pressure,[29] and hypertension does not appear to contribute to endothelial dysfunction in ACE2-deficient mice. We did not find any significant differences in blood pressure between WT and ACE2 KO mice in either age group. Our values for blood pressures are comparable with those reported previously for ACE2 KO mice.[25] These findings in mice are consistent with studies in humans, in which association of ACE2 polymorphisms with hypertension is variable.[30–32]

We found that endothelial function was impaired in adult ACE2 KO mice. Moreover, we found that cerebrovascular dysfunction during aging was augmented in old ACE2 KO mice. Because a superoxide scavenger restored endothelial responses to ACh, our data suggest that oxidative stress plays a primary role in dysfunction caused by ACE2 deficiency. Furthermore, nitrotyrosine staining appeared to be higher in basilar arteries from ACE2 KO mice, which suggest that these vessels are exposed to relatively greater oxidative stress than WT mice. Consistent with these findings, superoxide has been proposed to be a key mediator of cerebrovascular dysfunction in other models of aging and disease.[6,7,12,13,33–35]

ACE2 may play an important role in regulation of oxidative stress in blood vessels. ACE2 overexpression prevents angiotensin II–induced increase in ROS and NADPH oxidase expression in endothelium.[26] However, ACE2 inhibition enhanced angiotensin II–stimulated ROS formation.[36] We measured expression of antioxidant proteins, NADPH oxidase subunits, and proinflammatory genes to explore possible mechanisms that may contribute to increased dysfunction in ACE2 KO mice. Concordant with previous studies,[6,7] we found

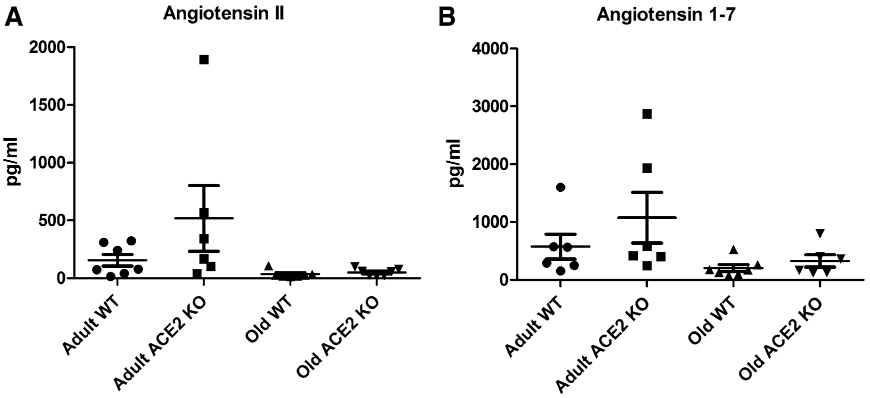

Figure 4. Plasma levels of angiotensin peptides. **A**, Angiotensin II levels in plasma from adult wild-type (WT; n=7), adult angiotensin-converting enzyme type 2 (ACE2) knockout (KO; n=6), old WT (n=7) and old ACE2 KO (n=7) mice. **B**, Angiotensin 1–7 levels in plasma from adult WT (n=6), adult ACE2 KO (n=6), old WT (n=6), and old ACE2 KO (n=7) mice. There were no significant differences in plasma peptide levels between groups. Values are mean±SE.

that aging increased expression of NADPH oxidase subunits in cerebral arteries. In addition, gene expression data indicated that aging had a significant effect on the expression of the proinflammatory molecules tumor necrosis factor-α and Rcan 1. Rcan1 modulates vasomotor function,[37] and its expression is increased by angiotensin II.[38] We also found an increase in SOD and catalase mRNA levels in cerebral arteries from old mice, which presumably is a compensatory mechanism to limit increases in ROS. We did not find changes in Nuclear factor (erythroid-derived 2)-like 2, which is thought to be a master regulator of antioxidant expression during aging. Our findings for antioxidant enzymes and Nuclear factor (erythroid-derived 2)-like 2 in cerebral vessels differ from previous studies in which decreased activity and expression of antioxidant proteins were found in rat aorta[39] and carotid arteries from macaques.[40]

We initially speculated that expression of ACE2 decreases with aging. which, by loss of inhibitory effects on the renin angiotensin system, might lead to higher concentrations of angiotensin II and more angiotensin II–related cardiovascular pathology with aging. Multiple lines of evidence suggest an association between increased angiotensin II signaling and aging. First, AT1R-deficient mice live longer than WT controls.[41] Second, cerebrovascular dysfunction with aging is attenuated in AT1R-deficient mice.[8] Third, long-term administration of AT1R blockers is associated with improved metabolic profiles during aging, which mimic some of the effects of caloric restriction.[42] We did not find, however, an effect of aging on expression of ACE2, Mas, or AT1R in cerebral arteries, kidney, or brain cortex. These results differ from findings in rat lung, in which expression of ACE2 protein decreases with aging.[24]

We used mice in which ACE2 is knocked out in all tissues. Contrary to what could be expected, we did not find differences in plasma levels of angiotensin II or angiotensin 1–7 between ACE2 KO and WT mice. These results agree with previous studies that demonstrated that plasma angiotensin II or angiotensin 1–7 were not significantly different in plasma from ACE2-deficient and WT mice.[43,44] It is possible that other enzymes, including prolyl[45] or neutral endopeptidases,[46] compensate for ACE2 deficiency and maintain normal angiotensin 1–7 levels. Moreover, ACE2 metabolizes several peptides, including angiotensin II, apelin, des-Arg^9 bradykinin, ghrelin, and neurotensins.[18] Some of these peptides modulate vasomotor function, so it is possible that the altered vascular phenotype in ACE2 KO mice can be explained by alterations in signaling pathways other than (or in addition to) the angiotensin II pathway.

In summary, this is the first study to show that ACE2 deficiency impaired function in cerebral arteries and exaggerates cerebrovascular dysfunction with aging. It is known that angiotensin II impairs neurovascular coupling,[12] induces oxidative stress and produces vasomotor dysfunction in cerebral arteries,[15,16] and plays an important role in cerebrovascular dysfunction with aging.[8] Therefore, it is possible that by modulating the effects of angiotensin II, ACE2 plays an important role in the maintenance of vascular function and prevention of cerebrovascular disease. Therapeutic approaches to increase ACE2 levels and activity might be beneficial in the management and prevention of cerebrovascular disease.

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Disclosures

None.

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