Brain Angiotensin-Converting Enzyme Type 2 Shedding Contributes to the Development of Neurogenic Hypertension

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Rationale: Overactivity of the brain renin–angiotensin system is a major contributor to neurogenic hypertension. Although overexpression of angiotensin-converting enzyme type 2 (ACE2) has been shown to be beneficial in reducing hypertension by transforming angiotensin II into angiotensin-(1–7), several groups have reported decreased brain ACE2 expression and activity during the development of hypertension.

Objective: We hypothesized that ADAM17-mediated ACE2 shedding results in decreased membrane-bound ACE2 in the brain, thus promoting the development of neurogenic hypertension.

Methods and Results: To test this hypothesis, we used the deoxycorticosterone acetate–salt model of neurogenic hypertension in nontransgenic and syn-hACE2 mice overexpressing ACE2 in neurons. Deoxycorticosterone acetate–salt treatment in nontransgenic mice led to significant increases in blood pressure, hypothalamic angiotensin II levels, inflammation, impaired baroreflex sensitivity, and autonomic dysfunction, as well as decreased hypothalamic ACE2 activity and expression, although these changes were blunted or prevented in syn-hACE2 mice. In addition, reduction of ACE2 expression and activity in the brain paralleled an increase in ACE2 activity in the cerebrospinal fluid of nontransgenic mice after deoxycorticosterone acetate–salt treatment and were accompanied by enhanced ADAM17 expression and activity in the hypothalamus. Chronic knockdown of ADAM17 in the brain blunted the development of hypertension and restored ACE2 activity and baroreflex function.

Conclusions: Our data provide the first evidence that ADAM17-mediated shedding impairs brain ACE2 compensatory activity, thus contributing to the development of neurogenic hypertension. (Circ Res. 2013;113:1087-1096.)

Key Words: angiotensin • autonomic dysfunction • baroreflex • central nervous system • gene therapy • hypertension • inflammation

The brain renin–angiotensin system (RAS) plays a critical role in the regulation of blood pressure (BP).1,2 Angiotensin II (Ang-II), the main peptide in this system, by acting on brain Ang-II type 1 receptors (AT\(_R\)), can increase sympathetic outflow, BP, water intake, salt appetite, and vasopressin release, but it also decreases baroreflex gain and vagal tone. Overactivity of the RAS is a major contributor to the development and maintenance of neurogenic hypertension in experimental models and humans, and RAS blockers are the most commonly prescribed drugs for the treatment of high BP.3

Angiotensin-converting enzyme type 2 (ACE2) was discovered a decade ago and has been recognized as a critical component of the RAS.4,5 We previously identified the presence of ACE2 in the mouse brain,6 notably in regions involved in the control of cardiovascular function, supporting a potential role for ACE2 in central BP regulation. By transforming Ang-II into the vasodilatory peptide angiotensin-(1–7) [Ang-(1–7)], ACE2 not only reduces AT\(_R\) stimulation by Ang-II but also promotes Mas receptor (MasR) activation by Ang-(1–7), leading to nitric oxide release7 and opposing the development of neurogenic hypertension.3

To study the impact of ACE2/Ang-(1–7)/MasR axis of RAS in hypertension, overexpression of ACE2 has been used extensively in animal models. Studies from various groups, including ours, reported that ACE2 overexpression leads to a reduction in sympathetic drive, an improvement in baroreflex gain, and, overall, a reduction of BP in experimental hypertension models.7,11 However, fewer studies addressed the effects of RAS overactivation on endogenous ACE2 in neurogenic hypertension. In spontaneously hypertensive rats, ACE2 protein expression was shown to be reduced in the rostral ventrolateral medulla, a region containing presympathetic neurons responsible for the maintenance of hypertension.11 Similarly, we previously observed reduced ACE2 activity in the brain stem of genetically hypertensive mice with chronic elevation of Ang-II.8 These findings suggest that although ACE2...
overexpression is beneficial in reducing high BP, its compensatory effects are blunted during the development of hypertension. Moreover, the mechanisms involved in the reduction of endogenous brain ACE2 during the development of neurogenic hypertension are unknown.

A process called ACE2 shedding, during which the ACE2 ectodomain is cleaved from the cell membrane and released into the extracellular milieu, was previously reported in vitro. This shedding is mediated by a member of the disintegrin and metalloproteinase family, ADAM17. However, the impact of ACE2 shedding has not been studied in neurogenic hypertension. Our study aimed at testing the hypothesis that chronic RAS activation enhances ADAM17 activity and promotes ACE2 shedding, leading to ACE2 secretion and thereby preventing its compensatory role in neurogenic hypertension.

Our data show that ACE2 shedding is activated in the brain of deoxycorticosterone acetate (DOCA)-salt hypertensive mice, promoting the reduction of membrane-bound ACE2 in these animals. In addition, using transgenic mice, we showed that maintaining high levels of ACE2 specifically in neurons (SA), we previously observed that high ACE2 activity in the brain prevents the development of neurogenic hypertension and knockdown of ADAM17 in the brain prevents ACE2 shedding and DOCA-salt–induced hypertension.

**Methods**

A detailed Methods section is available in the Online Data Supplement.

**Transgenic Mice and Animal Husbandry**

Experiments were performed in adult male (14–16 weeks old; 25–30 g) transgenic synapsin-hACE2 (SA) mice and nontransgenic (NT) littermates. SA mice were generated in collaboration with Dr Curt D. Sigmund at the University of Iowa and backcrossed into the C57Bl/6 background for >9 generations. Animals were housed in a temperature-controlled and humidity-controlled facility under a 12-hour dark/light cycle and fed standard mouse chow and water ad libitum. All procedures were approved by the Louisiana State University Health Sciences Center–New Orleans Animal Care and Use Committee and are in agreement with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**DOCA-Salt Treatment and Physiological Recordings**

Baseline BP was recorded by telemetry in uninephrectomized SA and NT cohorts for 3 days. Then, mice were randomly divided into 4 groups (n=12 per group), implanted subcutaneously with either a DOCA silicone (DOCA:silicone, 1:3; DOCA, 1 mg/g body weight) or an empty silicone (sham surgery) sheet. Drinking water from DOCA-implanted mice was replaced by 1% NaCl solution. BP was continuously recorded for 3 additional weeks. At the end of the protocol, the mice were euthanized and the brains and plasma were collected for enzyme activity assays, peptide level measurements, immunoprecipitation, and Western blot analysis. In another set of experiments, uninephrectomized NT mice were divided into 4 groups (n=6 per group) and infused intracerebroventricularly (icv) for 3 weeks with either ADAM17 siRNA (0.1 nmol/d) or artificial cerebrospinal fluid (CSF) while receiving DOCA-salt or sham treatment. BP was recorded daily by radiotelemetry as described. Another subset of NT mice was infused with losartan (2 µg/h icv) for 3 weeks while receiving DOCA-salt treatment. Spontaneous baroreceptor reflex sensitivity (SBRSS), reflecting the baroreflex control of heart rate, was calculated using the sequence method described previously. Autonomic function was assessed in conscious freely moving mice before and 3 weeks after DOCA-salt treatment using a pharmacological method involving intraperitoneal injection of propranolol (β-blocker; 4 mg/kg), atropine (muscarinic receptor blocker; 1 mg/kg), and chlorisondamine (ganglionic blocker; 5 mg/kg). Each injection was separated by at least a 3-hour recovery period. Changes in heart rate or mean arterial pressure (MAP) were calculated after administration of these blockers. At the end of the protocol, mice were euthanized and the brains and plasma were collected and stored at −80°C until used in the various assays.

**Statistics**

Data are presented as mean±SEM. Data were analyzed by repeated measures ANOVA or 2-way ANOVA, followed by Bonferroni post tests for multiple comparisons between means, as appropriate. Statistical comparisons were performed using Prism 5 (GraphPad Software). Differences were considered statistically significant at P<0.05.

**Results**

**Brain ACE2 Overexpression Reduces DOCA-Salt–Induced Hypertension by Preserving Baroreflex Sensitivity and Autonomic Function**

Using a transgenic mouse model overexpressing ACE2 specifically in neurons (SA), we previously observed that high ACE2 activity in the brain prevents the development of hypertension induced by chronic Ang-II infusion. Although this model is widely used, it is not representative of the type of hypertension associated with salt sensitivity and low renin levels, which is common in African Americans and patients with type II diabetes. To address the ability of neuronal ACE2 to overcome RAS overactivity in these conditions, we developed DOCA-salt hypertension, a model of neurogenic hypertension, in both NT and SA mice. ACE2 overexpression in the brain did not affect any of the baseline hemodynamic and cardiovascular parameters, such as MAP, SBRSS, or autonomic function (Figure 1A–E). DOCA-salt treatment produced a progressive increase in MAP in both NT and SA mice, which reached a plateau by the end of the first week. However, ACE2 overexpression in SA plus DOCA mice was associated with a significantly smaller increase in MAP than in NT plus DOCA mice (P<0.05; Figure 1A). These data confirm that, as reported in the Ang-II infusion model, brain
ACE2 expression can significantly impair the development of neurogenic hypertension.

The baroreceptor reflex is the main mechanism involved in the beat-to-beat maintenance of BP within a normal range, and its sensitivity is often impaired in hypertension, for example, as a result of elevated Ang-II levels. To assess the effects of neuronal ACE2 on baroreflex function, we determined SBRS using the sequence method. As expected, SBRS was significantly reduced in NT plus DOCA mice compared with sham (P<0.05; Figure 1B). However, SA mice showed no impairment of SBRS during DOCA-salt treatment (Figure 1B).

A balance between sympathetic drive and vagal tone is also critical for BP regulation. Autonomic function was assessed in conscious mice after 3 weeks of treatment after randomized injections of sympatholytic drugs and a vagal blocker. Autonomic function was identical between sham-treated NT and SA mice (Figure 1C–E). DOCA-salt administration resulted in significant increases in both cardiac and vascular sympathetic drive (P<0.05 versus sham; Figure 1C and 1D), whereas vagal tone was blunted (P<0.05 versus vehicle; Figure 1E), contributing to the maintenance of hypertension in NT plus DOCA mice. However, cardiac dysautonomia was prevented and sympathetic drive to the vasculature was reduced in SA plus DOCA mice (Figure 1C–E).

These data provide strong evidence that ACE2 expression in neurons plays a critical role in maintaining the functionality of baroreflex and autonomic regulations during the development of hypertension.

Figure 1. Neuron-targeted angiotensin-converting enzyme type 2 (ACE2) overexpression attenuates deoxycorticosterone acetate (DOCA)-salt hypertension. DOCA implanted subcutaneously and combined with 1% saline drinking solution induced a progressive increase of mean arterial pressure (MAP; A) in uninephrectomized (n=12 mice per group) nontransgenic (NT) and syn−hACE2 (SA) mice with neuron-specific expression of human ACE2. After 21 days of DOCA-salt treatment, spontaneous baroreceptor reflex sensitivity (SBRS; B) was calculated using the sequence method, and autonomic function was assessed pharmacologically by determining the changes in MAP (ΔMAP) and heart rate (ΔHR) after intraperitoneal injections of a β-blocker (propranolol, 4 mg/kg; C), ganglionic blocker (chlorisondamine, 5 mg/kg; D), and muscarinic antagonist (atropine, 1 mg/kg; E). At the end of the protocol, hypothalamic angiotensin II (F), plasma arginine vasopressin (AVP; G), and urinary norepinephrine (H) were determined using enzyme-linked immunosorbent assay kits (n=3–6 mice per group). *P<0.05 vs sham; †P<0.05 vs NT plus DOCA.

ACE2 Overexpression Prevents DOCA-Salt–Mediated RAS Overactivity

ACE2 has been recognized as a member of the RAS, because it transforms Ang-II into the vasodilatory peptide Ang-(1–7), thus providing a double opportunity to oppose the overactive RAS. Brain Ang-II is known to increase norepinephrine and arginine vasopressin (AVP) release, thereby contributing to the maintenance of hypertension. We tested whether ACE2 expression could regulate the release of these prohypertensive neuropeptides by modulating Ang-II levels in the brain. Measurement of Ang-II levels using enzyme-linked immunosorbent assay in NT plus DOCA mice revealed a 3-fold increase in the hypothalamus (P<0.05 versus NT plus sham; Figure 1F) but not in the plasma (NT plus sham, 23±1 pg/mL; NT plus DOCA, 20±1 pg/mL), confirming that DOCA-salt hypertension results in enhanced brain Ang-II levels without altering the systemic RAS. Similarly, plasma AVP (P<0.05 versus NT plus sham; Figure 1G) and urinary norepinephrine (P<0.05 versus NT plus sham; Figure 1H) levels were significantly increased after DOCA-salt treatment. ACE2 overexpression in the brain did not alter the baseline levels of these peptides (Figure 1F–H). However, it prevented the DOCA-salt–induced increase in hypothalamic Ang-II levels (P<0.05 versus NT plus DOCA; Figure 1F). Moreover, it was associated with a 50% reduction of AVP levels (Figure 1G; P<0.05 versus NT plus DOCA; P<0.05 for interaction between DOCA treatment and genotype) and prevented the increase in urinary norepinephrine levels (P<0.05 versus NT plus DOCA; Figure 1H).

Although elevation of brain Ang-II levels has been reported to mediate an inflammatory response before the development of hypertension, the contribution of Ang-II in this process has been questioned in DOCA-salt hypertension. Therefore, we extended our study by measuring tumor necrosis factor (TNF)-α, IL-1β, IL-6, and MCP-1 in the brain during DOCA-salt hypertension. Reverse-transcription polymerase chain reaction shows that mRNA for all proinflammatory cytokines and chemokine was increased in the hypothalamic paraventricular nucleus (PVN) of NT plus DOCA mice (Figure 2A–D), confirming the feed-forward mechanism in DOCA-salt hypertension. Moreover, these increases were significantly blunted (TNF-α, IL-1β, and MCP-1) or prevented (IL-6) in SA plus DOCA mice, supporting the beneficial effects of ACE2 expression in the prevention of RAS-mediated inflammation.
AT1R and MasR mRNA and protein levels were assessed by whether these were affected in DOCA-salt hypertension. Indeed, ACE2, Ang-(1–7), and the MasR, we tested as well as reduction of components of the compensatory proinflammatory cytokines (IL-1β, IL-6, and tumor necrosis factor) and chemokine (MCP-1) in hypothalamic paraventricular nucleus samples isolated from nontransgenic (NT) and syn-hACE2 (SA) mice after a 3-week DOCA-salt, or sham, treatment. Data are means±SEM (n=3 per group). *P<0.05 vs sham; †P<0.05 vs NT plus DOCA.

Because RAS overactivity is associated with increased levels of classic RAS components such as Ang-II and AT1R, as well as reduction of components of the compensatory RAS such as ACE2, Ang-(1–7), and the MasR, we tested whether these were affected in DOCA-salt hypertension. AT1R and MasR mRNA and protein levels were assessed by quantitative reverse-transcription polymerase chain reaction and Western blotting. In NT mice, DOCA-salt treatment resulted in upregulation of AT1R and a concomitant reduction of MasR levels in the PVN (Figure 3A–D), contributing to RAS overactivity. Conversely, ACE2 overexpression was associated with MasR upregulation and prevented the receptor changes mediated by DOCA-salt treatment (Figure 3; P<0.05 for interaction between DOCA treatment and genotype). To further establish brain RAS overactivity, a subset of NT mice was chronically infused with AT1R blocker losartan (icv) while treated with DOCA-salt for 3 weeks. Online Figure I shows that brain AT1R blockade prevented the development of neurogenic hypertension, confirming the critical role of central AT1R in this model. Together, our data show that DOCA-salt hypertension leads to brain RAS overactivity and increase in prohypertensive peptides and proinflammatory molecules and is mediated by enhanced AT1R and reduction of MasR levels. In addition, our data strongly support the pivotal role of ACE2 in the catabolism of Ang-II in the brain, thereby reducing RAS overactivity.

**ADAM17 Stimulates ACE2 Shedding in the Brain of DOCA-Salt Hypertensive Mice**

The findings presented suggest that ACE2 expression can exert an inhibitory role on RAS overactivity, thus abating the development of hypertension. However, we and others previously showed that RAS overactivity is associated with downregulation of brain endogenous ACE2 in genetic models of hypertension. We tested whether endogenous brain ACE2 activity/protein is altered during DOCA-salt hypertension. In NT plus DOCA mice, ACE2 activity was reduced by 50% in the hypothalamus (P<0.05 versus NT plus sham; Figure 4A). To determine whether these changes are restricted to ACE2 enzymatic activity, mRNA and protein expression were analyzed in the hypothalamus using species-specific primers and an antibody specific for mouse ACE2. Although transcriptional regulation was ruled out by unaffected ACE2 mRNA levels (Online Figure II), ACE2 protein expression was similarly reduced by DOCA-salt treatment in both genotypes, albeit more modestly than activity (Figure 4B). To further clarify ACE2 regulation, we performed microdialysis and assessed ACE2 activity in the CSF. Surprisingly, NT plus DOCA mice exhibited a 2-fold increase in ACE2 activity in the CSF (P<0.05 versus NT plus sham; Figure 4C), indicating that the ACE2 ectodomain might be shed from the plasma membrane. ADAM17 was previously reported to promote ACE2 shedding in vitro. The involvement of this sheddase was confirmed in NT plus DOCA mice by the observation of increased ADAM17 activity and expression in the hypothalamus (P<0.05 versus NT plus sham; Figure 4D and 4E). To confirm that increased ADAM17 is mediated by RAS overactivity, protein expression was determined in

![Figure 2. Angiotensin-converting enzyme type 2 overexpression reduces deoxycorticosterone acetate (DOCA)-salt–induced inflammation.](image)

![Figure 3. Neuron-specific angiotensin-converting enzyme type 2 overexpression reverses the deoxycorticosterone acetate (DOCA)-salt–induced changes in angiotensin receptors.](image)
losartan-treated hypothalami. Online Figure III shows that blockade of AT\(_1\)R prevented the increase in ADAM17 expression in DOCA-salt–treated mice. Because calmodulin (CaM) binding to the cytoplasmic tail of ACE2 has been described as a protective mechanism against shedding,\(^{20}\) we then assessed CaM-bound ACE2 levels after immunoprecipitation. Interestingly, we observed a significant reduction of CaM-bound ACE2 in DOCA-salt–treated NT mice (\(P<0.05\) versus NT plus sham; Figure 4F), consistent with the idea that unprotected ACE2 might have become a target for shedding. Together, our data provide strong evidence in favor of a RAS overactivity-mediated mechanism leading to enhanced ADAM17 expression and activity that participates in ACE2 downregulation through shedding of its catalytic site from the plasma membrane into the surrounding milieu.

Moreover, our data show that in SA mice the enhanced ACE2 activity in the central nervous system was unaffected by DOCA-salt treatment. It appears that ACE2 overexpression can limit the impact of ADAM17-mediated shedding and overcome the feed-forward mechanism of RAS overactivity.

**Knockdown of ADAM17 Attenuates DOCA-Salt–Induced Hypertension**

These findings suggest that RAS overactivity leads to upregulation of ADAM17, resulting in enhanced ACE2 shedding and the maintenance of hypertension. To determine whether ADAM17 contributes to DOCA-salt hypertension, we hypothesized that ADAM17 knockdown would prevent the development of high BP. Because constitutive deletion of ADAM17 is associated with developmental problems, and because antagonists lack specificity, we relied on siRNA methodology. To validate this approach, a cocktail of ADAM17-targeted siRNA was first tested in a neuroblastoma cell line. Neuro2A cells incubated with Ang-II (100 nmol/L, 24 hours) showed a significant increase in ADAM17 expression, confirming the ability of Ang-II to upregulate this sheddase (Online Figure IV). However, pretreatment with ADAM17 siRNA, but not scrambled siRNA, blocked \(\approx 90\%\) of ADAM17 expression. This validation being achieved, we infused ADAM17 siRNA centrally (0.1 nmol/day icv) in NT mice during DOCA-salt administration. A 3-week icv infusion of ADAM17 siRNA had no effect on MAP or SBRS levels in sham-treated mice (\(P>0.05\) versus NT plus sham; Figure 5A and 5B). Interestingly, knockdown of ADAM17 in the brain significantly attenuated DOCA-salt–induced hypertension (\(P<0.05\) versus NT plus DOCA; Figure 5A). As one of the mechanisms that might have contributed to the reduction of hypertension, SBRS was restored to the normal values observed in NT plus sham mice (Figure 5B). In addition, Western blotting shows that ADAM17 siRNA infusion resulted in a significant reduction of ADAM17 expression in the hypothalamus compared with the increase in ADAM17 expression in NT plus DOCA mice (Figure 5C; \(P<0.05\) for interaction between DOCA treatment and siRNA infusion). The improvements of BP and SBRS in these DOCA-salt mice were concomitant to a \(\approx 50\%\) knockdown of ADAM17 expression (Figure 5C), which was associated with a blunted reduction of ACE2 activity in the
hypothalamus and normalization of ACE2 activity in the CSF (Figure 5D). These data confirm that ADAM17 contributes to the maintenance of DOCA-salt hypertension by impairing ACE2 compensatory activity.

Discussion

Since the discovery of ACE2 in 2000, as the missing link between the classic RAS and its compensatory axis,1 many reports have supported the pivotal role of this enzyme in cardiovascular diseases. However, although overexpression studies have established the benefits of ACE2, very little has been accomplished to understand the regulation of this carboxypeptidase. Accordingly, we first investigated whether brain ACE2 could play a therapeutic role in a poorly studied but clinically relevant high BP model—the DOCA-salt hypertension. The next objective was to clarify the role of brain ACE2 shedding in the regulation of the compensatory activity of the enzyme. Finally, we assessed whether prevention of ACE2 shedding could affect the development of DOCA-salt hypertension. Our data, for the first time to our knowledge, provide strong evidence that low-renin hypertension is associated with reduced ACE2 expression and activity in the brain, leading to autonomic dysfunction, impaired baroreflex sensitivity, inflammation, and hypertension. In addition, we show that Ang-II–mediated upregulation of ADAM17 contributes to ACE2 shedding, thus reducing the membrane-bound carboxypeptidase levels and increasing its soluble form. Finally, we demonstrate that knockdown of ADAM17 prevents the reduction of ACE2 levels in the brain and is associated with a blunting of DOCA-salt hypertension. Therefore, we have established for the first time that ACE2 shedding contributes to the development of neurogenic hypertension.

Low-renin hypertension accounts for 25% of patients with high BP. Previous studies have demonstrated that DOCA-salt hypertension, a low-renin hypertension model,12–14 is mediated through activation of the RAS within the brain and is associated with enhanced sympathetic activity and vasopressin secretion.25,26 One of the many brain regions involved in the central regulation of BP is the hypothalamic PVN, which contains both sympathetic and vasopressinergic neurons. In addition, we previously reported that ACE2 is present in this region and capable of modulating cardiovascular function.6,9 In our study, although there was no alteration in plasma Ang-II levels, we observed overactivity of the classic RAS (Ang-II and AT1R) in the PVN and a simultaneous impairment of the compensatory RAS (ACE2 and MasR). These alterations could be prevented by ACE2 overexpression in SA mice, suggesting a role for locally produced Ang-II in mediating the hypertensive response and as evidenced by the reduction of hypothalamic Ang-II levels in SA mice. Moreover, brain AT1R blockade prevented the development of DOCA-salt hypertension, confirming the critical role of central AT1R in this model of neurogenic hypertension. These data suggest that low-renin hypertension, like other forms of neurogenic hypertension,7,8 is detrimental for the compensatory RAS in the brain, leading to downregulation of the compensatory axis and exacerbation of the classic RAS, altogether reinforcing a feed-forward mechanism promoting dysautonomia, release of vasoactive peptides, and ultimately elevating BP. Although we did not directly assess the contribution of Ang-(1–7) in this model during DOCA-salt hypertension, we previously reported that SA mice have an altered Ang-II:Ang-(1–7) ratio in favor of the vasodilatory peptide and that their resistance to neurogenic hypertension is mediated by Ang-(1–7) via its MasR.7

Previous studies have supported the interaction between centrally acting Ang-II and the systemic release of AVP.27,28 DOCA-salt treatment increases the release of AVP,25 which in turn strongly contributes to the pressor effect of centrally acting Ang-II.24,28 Interestingly, Ang-(1–7), the product of Ang-II degradation by ACE2, was originally reported to promote AVP release from hypothalamic explants.29 However, our in vivo studies argue against this hypothesis. In our study, the formation of Ang-(1–7) in the brain of SA mice, previously reported as a contributing mechanism to the reduction of neurogenic hypertension,7 was associated with a reduction
of AVP release; therefore, it is unlikely that the heptapeptide could participate in AVP release in vivo. We speculate that ACE2 overexpression in the brain leads to the conversion of Ang-II into Ang-(1–7), thus preventing Ang-II stimulation of AVP release and thereby contributing to the reduction of neurogenic hypertension.

In recent years, evidence has accumulated that hypertension is a chronic low-grade inflammatory condition and that activation of proinflammatory mechanisms contributes to the pathogenesis of hypertension. A recent study showed that Ang-II–mediated hypertension is caused by central mechanisms and described a feed-forward process.

![Diagram of the renin-angiotensin system (RAS), ADAM17, and neurogenic hypertension](image)

**Figure 6.** Brain renin–angiotensin system (RAS), ADAM17, and neurogenic hypertension. On baseline, angiotensin II (Ang-II) is formed from Ang-I by the angiotensin-converting enzyme (ACE) and binds Ang-II type 1 (AT1R) and type 2 (AT2R) receptors. Ang-II can be cleaved by ACE2 to form Ang-(1–7), which then interacts with the Mas receptor (MasR). RAS overactivity under deoxycorticosterone acetate (DOCA)-salt stimulation results in increased levels of Ang-II and AT1R expression, leading to increased expression of ADAM17, which in turn cleaves ACE2, resulting in decreased membrane ACE2 levels, thereby decreasing Ang-(1–7) formation, reducing MasR activation, and ultimately contributing to the development of neurogenic hypertension. Overexpression of ACE2 in the brain results in enhanced conversion of DOCA-salt–induced increase in Ang-II levels, thereby promoting enhanced formation of Ang-(1–7) levels while inhibiting ADAM17 upregulation.
in which the central pressor effects of Ang-II lead to activation of T cells, which in turn promote vascular inflammation and further increase BP, leading to severe hypertension. Despite previous reports showing increased proinflammatory cytokines in the heart, kidneys, and aorta in DOCA-salt hypertension, a recent study challenged the participation of these molecules in the brain. Our study confirmed previous observations made in peripheral tissues and extended the findings to the PVN, showing that DOCA-salt-induced hypertension is associated with elevated proinflammatory cytokines and chemokines in the brain, as evidenced by an increase in TNFα, IL-1β, IL-6, and MCP-1. Brain-specific overexpression of ACE2 attenuated the increase in local tissue Ang-II levels and inflammation, thereby providing an additional mechanism by which ACE2 contributes to BP regulation.

The present study provides strong evidence for ACE2 shedding as a novel mechanism involved in the development of neurogenic hypertension (Figure 6). Our data show that DOCA-salt hypertension was associated with increased ADAM17 expression and activity in the hypothalamus, whereas membrane ACE2 expression was reduced and soluble ACE2 was increased in the CSF. Moreover, we observed that treatment of neurons with Ang-II resulted in upregulation of ADAM17, consistent with previous reports showing that EGF receptor transactivation is mediated by Ang-II stimulation of ADAM17. The fact that ACE2 shedding could be reduced by ACE2 overexpression suggests that Ang-II is a major player in the activation of ADAM17 in DOCA-salt hypertension (Figure 6). Mouse ACE2 mRNA level was not altered in NT and SA mice with or without DOCA-salt treatment, confirming that the reduced ACE2 activity in DOCA mice is posttranslational and not at the transcriptional level, consistent with our hypothesis that ACE2 shedding could be a major mechanism involved. ADAM17 is thought to exist as a latent protein in the cytoplasm, which on activation by reactive oxygen species is translocated to the plasma membrane, where it can exert its shedding process. Although ADAM17, also called TNFα convertase, can promote the shedding of multiple membrane proteins, including TNFα, its receptor, and the EGF receptor, it is the only sheddase known to cleave ACE2, although the exact site of cleavage remains to be determined. ADAM17-mediated ACE2 shedding has previously been reported in cell cultures, where cleavage of the membrane-bound ACE2 releases a soluble form that retains its carboxypeptidase activity. In addition, ACE2 shedding was recently suggested as a biomarker for chronic kidney disease. ADAM17 has been suggested as a player in vascular remodeling, hypertrophy, and growth processes, mostly by promoting transactivation of the EGF receptor, but it has never been shown to directly modulate BP. Therefore, ACE2 shedding provides the first direct evidence for a role of ADAM17 in BP regulation. This hypothesis is supported by our data showing that ADAM17 knockdown was associated with reduction of ACE2 shedding, restoration of baroreflex function, and a decrease in DOCA-salt hypertension, similar to what was observed in mice overexpressing ACE2 on neurons. In addition, targeting of ADAM17 in hypertension provides multiple benefits because it would allow for the reduction of active TNFα levels, thus reducing part of the inflammatory component and preventing ACE2 shedding, thereby preserving the compensatory function of this carboxypeptidase.

Interestingly, our study also identified weaknesses among the mechanisms supposed to protect ACE2 function from shedding. CaM was previously reported to bind the cytoplasmic tail of ACE2 and thereby confers protection against ADAM17-mediated shedding. However, our data show that in DOCA-salt hypertension ACE2-CaM binding was reduced, leaving the carboxypeptidase unprotected and more sensitive to ADAM17-mediated shedding. Interestingly, this could not be rescued by ACE2 overexpression, suggesting that RAS overactivity might not be directly involved in reducing the ACE2–CaM interaction. It is possible that changes in protein conformation resulting from increased osmolality might have altered the protein binding, and this needs to be further addressed.

In conclusion, our data, for the first time, provide strong evidence that low-renin hypertension is associated with reduced ACE2 expression and activity in the brain, leading to autonomic dysfunction, impaired baroreflex sensitivity, inflammation, and hypertension. In addition, we show that brain Ang-II–mediated upregulation of ADAM17 contributes to ACE2 shedding, thus reducing the membrane-bound carboxypeptidase levels and increasing its soluble form (Figure 6). Finally, we demonstrate that knockdown of ADAM17 prevents the reduction of ACE2 levels in the brain and is associated with a reduction of DOCA-salt hypertension. Therefore, we have established for the first time that ACE2 shedding contributes to the development of neurogenic hypertension.

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Disclosures

None.

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**Novelty and Significance**

**What Is Known?**

- Overactive brain renin–angiotensin system (RAS) is associated with decreased angiotensin-converting enzyme 2 (ACE2) level and activity in the brain during the development of neurogenic hypertension.
- ADAM17 is a member of the disintegrin and metalloproteinase family that has been shown to target ACE2 for cleavage in vitro.

**What New Information Does This Article Contribute?**

- ADAM17 level and activity were increased in the brain in deoxycorticosterone acetate (DOCA)-salt–induced hypertensive mice.
- ACE2 overexpression in the brain attenuates DOCA-salt–induced hypertension by reversing changes in angiotensin II (Ang-II) type 1 receptor and Mas receptor and reducing ADAM17 level in the brain.
- ADAM17 knockdown in the brain during the course of DOCA-salt hypertension is associated with restoration of ACE2 level and activity as well as a reduction of high blood pressure.

Since the discovery of ACE2 in 2000 as the missing link between the classic RAS and its compensatory axis, ACE2 has emerged as a potential therapeutic target in a variety of cardiovascular diseases. However, regulation of ACE2 and the mechanisms leading to its inhibition remain largely unknown. In this study, for the first time to our knowledge, we provide strong evidence that low-renin hypertension is associated with reduced ACE2 expression and activity in the brain, leading to autonomic dysfunction, impaired baroreflex sensitivity, inflammation, and hypertension. Increased Ang-II levels during DOCA-salt–induced hypertension results in upregulation of ADAM17, contributing to ACE2 shedding. The knockdown of ADAM17 prevents the reduction of ACE2 levels in the brain and blunts DOCA-salt–induced hypertension. Thus, we have shown that ACE2 shedding contributes to the development of neurogenic hypertension. Although our investigation focuses on the brain RAS, we speculate that the findings might be relevant to diseases featuring overactivity of the RAS.
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DETAILED METHODS

Transgenic mice and animal husbandry
Experiments were performed in adult male (14-16 weeks old, 25-30 g) transgenic syn-hACE2 (SA) mice and non-transgenic littermates (NT). SA mice were generated in collaboration with Dr. Curt D. Sigmund at The University of Iowa and back-crossed into the C57Bl/6 background for more than 9 generations. Animals were housed in a temperature- and humidity-controlled facility under a 12 hour dark/light cycle, fed standard mouse chow and water ad libitum. All procedures were approved by the LSU Health Sciences Center-NO Animal Care and Use Committee and are in agreement with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

DOCA-salt treatment and physiological recordings
For each surgery, mice were anesthetized with isoflurane (2%) in an oxygen flow (1 L/min) and placed on a heating pad to maintain body temperature. Post-operative care, included a buprenorphine injection to relieve pain at the end of the surgery and after 12 hours (0.05 mg/Kg, sc). SA and NT mice first underwent uninephrectomy surgery. An incision was made in the skin in the retro peritoneal region and the right kidney was removed. After 1 week of recovery, mice were implanted with telemetry probes for conscious blood pressure (BP) monitoring, as described. After another week of recovery, baseline blood pressure was recorded in SA and NT cohorts for 3 days, then mice were randomly divided into 4 groups (n=12/group), either implanted subcutaneously with a DOCA-silicone (DOCA:silicone=1:3; DOCA: 1 mg/g body weight) or an empty silicone (sham surgery) sheet. Drinking water from DOCA implanted mice was replaced by 1% NaCl solution. BP was continuously recorded for 3 additional weeks. At the end of the protocol, the mice were sacrificed, the brains and plasma were collected for enzyme activity assays, peptide level measurements, immuno-precipitation and western blot analysis. In another set of experiments, uni-nephrectomized NT mice were divided into 4 groups (n=6/group) and infused intracerebroventricularly (icv) for 3 weeks, with either ADAM17 siRNA (0.1 nmoles/day) or artificial cerebrospinal fluid (aCSF) while receiving DOCA-salt or sham treatment. Another subset of uni-nephrectomized NT mice were infused intracerebroventricularly (icv) for 3 weeks, with Losartan (2 µg/hour) while receiving DOCA-salt treatment. BP was recorded daily by radiotelemetry as described above. Spontaneous baroreceptor reflex sensitivity (SBRS), reflecting the baroreflex control of HR, was calculated using the sequence method as described. Autonomic function was assessed, in conscious freely moving mice, before and 3 weeks after DOCA-salt treatment, using a pharmacological method involving ip injection of propranolol (β-blocker, 4 mg/kg), atropine (muscarinic receptor blocker, 1 mg/kg) and chlorisondamine (ganglionic blocker, 5 mg/kg). Each injection was separated by at least a 3-hour recovery period. Changes in HR (ΔHR) or mean arterial pressure (ΔMAP) were calculated following administration of these blockers. At the end of the protocol, mice were euthanized and the brains and plasma were collected and stored at -80°C until used in the following assays.

Immunoprecipitation and western blotting
Protein (10-20 µg) extracted from hypothalami and paraventricular nucleus (PVN) were processed for Western blotting as described previously, using rabbit anti-mACE2 (Santa Cruz, sc-1:500), rabbit anti-ADAM17 (Abcam, ab2051; 1:1000), goat anti-AT1R (Santa Cruz, sc-9040; 1:1000) and goat anti-MasR (Alomone, 1:500) antibodies. Equal loading was determined using either α-actinin, α-tubulin or γ-tubulin loading controls. In some experiments, cell lysates from hypothalami were immuno-precipitated with an anti-calmodulin antibody (Catch and Release Reversible Immuno-precipitation System kit, UpState), following the manufacturer's instructions. Briefly, cell lysates (500 µg) were incubated with anti-calmodulin antibody (Abcam...
ab45689, 1:100), antibody capture affinity ligand (10 µl) and buffer. Eluates (10 µl) were then subjected to western blot analysis for mACE2 (1:500, Open Biosystem).

For in vitro experiments, Neuro2A cells (Mouse neuroblastoma, CCL-131, ATCC) were seeded (2×10^5 cells/well) and grown as described previously. After 24 hours, cells were transfected with ADAM17 siRNA and control siRNA using DharmaFECT transfection reagents (Thermo Scientific) according to the manufacturer’s instructions. After 48 hours of transfection, the cells were treated with Ang-II (100 nM) for an additional 24 hours in serum-free medium and harvested for western blotting analysis of ADAM17 expression as described above.

**Peptides bioassays**

Ang-II and vasopressin levels were measured from mouse hypothalami and/or plasma using Fluorescent EIA kits (FEK-002-12 and FEK-065-07, respectively, Phoenix Pharmaceuticals). Plasma and tissue homogenates were extracted using Sep-Pak C18 columns (Waters). The eluted fractions were dried using a vacuum centrifuge, re-suspended in assay buffer and used for EIA. Urine samples were collected at the end of the study by bladder massage and urinary norepinephrine concentration was measured with a Noradrenaline ELISA kit (Immuno-Biological Laboratories).

**Quantitative real time RT-PCR**

PVN punches were obtained from frozen brain sections (brain punch 1.0 mm, Stoelting). Total RNA was isolated from PVN or hypothalamic tissue using RNeasy plus micro kit (Qiagen), and a cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad). Real time RT-PCR amplification reactions were performed with iQ SYBR Green Super mix with ROX (Bio-Rad) using a Bio-Rad iQ5 Real time PCR machine (Bio-Rad). The primer sequences used for real time RT-PCR are listed in Supplemental Table 1. Data were normalized to β-actin expression by the ∆∆C_T comparative method and expressed as a fold change compared to sham.

**Microdialysis and ACE2 activity assay**

A sub-group of SA and NT mice (n= 4-6) was anesthetized with a Ketamine/xylazine mix (100 mg/kg /5 mg/kg I.P.), on Day 16 of the DOCA-salt treatment and implanted icv (1.0 mm lateral, 2.7 mm ventral , 0.3 mm caudal) with a cannula guide (CMA12). Five days later (on the 21st day of DOCA-salt treatment), a probe with a 100 kDa molecular weight cutoff membrane was inserted into the guide and microdialysis was performed in conscious freely moving mice. Mice were perfused continuously with artificial CSF (NaCl: 147, KCl: 4 and CaCl_2: 2.3 mM) at a rate of 1 µl/min (CMA 400 syringe pump) and the dialysate collected using a refrigerated fraction collector (CMA470). Dialysates were concentrated with a freeze dryer, re-suspended in ACE2 activity buffer at a 5X concentration, and used for ACE2 activity assay as described. Hypothalami collected from each group (n=5-7/group) were processed for ACE2 activity assay at the same time. Data are presented in arbitrary fluorescence units (AFU), as amounts of fluorescence substrate converted to product per minute and normalized for total protein or for volume of CSF.

**ADAM17 activity assay**

ADAM17 activity was measured in the hypothalamus (6 µg proteins/well) using a TACE activity kit (Sensolyte 520, ANASPEC), following the manufacturer’s instructions.

**Data analysis**

Data are presented as mean ±SEM. Data were analyzed by repeated measures ANOVA, or two-way ANOVA, followed by Bonferroni post-tests for multiple comparisons between means,
as appropriate. Statistical comparisons were performed using Prism5 (GraphPad Software). Differences were considered statistically significant at $P<0.05$.

References:


**Supplemental Table I.** List of primers used for real time RT-PCR

<table>
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<tr>
<th>Gene</th>
<th>Forward (5′-3′)</th>
<th>Reverse (5′-3′)</th>
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<tr>
<td>hACE2</td>
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ACE2, Angiotensin converting enzyme 2; AT₁R, Angiotensin II type 1 receptor; IL, Interleukin; MCP-1, Monocyte chemoattractant protein-1; TNF-α, Tumor necrosis factor-alpha.
Supplemental Figure I. Treatment with losartan prevents DOCA-salt induced hypertension. Three weeks of DOCA-salt treatment resulted in significant increase in mean arterial pressure (MAP) in uni-nephrectomized non-transgenic (NT) mice. Intracerebroventricular infusion of losartan (2 ug/hour) prevented the DOCA-salt mediated increase in blood pressure. Statistical significance: *P<0.05 vs. sham and †P<0.05 vs. NT+DOCA; n=6-12 mice/group.
Supplemental Figure II. Expression of mouse and human ACE2 mRNA. Quantitative real time RT-PCR measurements of mouse specific (mACE2) and human specific (hACE2) ACE2 mRNA in hypothalamus samples isolated from non-transgenic (NT) and syn-hACE2 (SA) mice following a 3-week DOCA-salt, or sham treatment. Data are means ±SEM (n=3/group). *P<0.05 vs. sham, †P<0.05 vs. NT+DOCA.
Supplemental Figure III. Treatment with losartan prevents DOCA-salt induced increase in ADAM17 expression. Three weeks of DOCA-salt treatment resulted in significant increase in hypothalamus ADAM17 protein expression in uni-nephrectomized non-transgenic (NT) mice. Intracerebroventricular infusion of losartan prevented the DOCA-salt mediated increase in ADAM17 expression. *P<0.05 vs. sham and †P<0.05 vs. NT+DOCA; n=6 mice/group.
Supplemental Figure IV. Validation of ADAM17 siRNA in neuro2A cells. Neuro2A cells were transfected for 48 hours with specific siRNA against ADAM17 before treatment with 100 nM angiotensin-II for 24 hours. A scrambled siRNA was used as negative control. (A) The protein extract was used for western blot analysis of ADAM17 protein expression. (B) Densitometric analysis of western blot data. Data are means ±SEM. Data are representative of 3 independent experiments (n=3/treatment). *P<0.05 vs. N2A+Vehicle, †P<0.001 vs. N2A+Ang II.