Brain ACE2 Shedding Contributes to the Development of Neurogenic Hypertension

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ABSTRACT

**Rationale:** Over-activity of the brain Renin Angiotensin System (RAS) is a major contributor to neurogenic hypertension. While over-expression of Angiotensin-Converting Enzyme type 2 (ACE2) has been shown to be beneficial in reducing hypertension by transforming Angiotensin (Ang)-II into Ang-(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 DOCA-salt model of neurogenic hypertension in non-transgenic (NT) and syn-hACE2 mice over-expressing ACE2 in neurons. DOCA-salt treatment in NT mice led to significant increases in blood pressure, hypothalamic Ang-II levels, inflammation, impaired baroreflex sensitivity, autonomic dysfunction, as well as decreased hypothalamic ACE2 activity and expression, while these changes were blunted or prevented in syn-hACE2 mice. In addition, reduction of ACE2 expression and activity in the brain paralleled a rise in ACE2 activity in the cerebrospinal fluid of NT mice following DOCA-salt treatment and was 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.

**Keywords:** Autonomic function, high blood pressure, central nervous system, gene therapy, inflammation, hypertension, renin angiotensin system, baroreflex control

**Nonstandard Abbreviations and Acronyms:**

<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>ACE2</td>
<td>Angiotensin converting enzyme type 2</td>
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<tr>
<td>aCSF</td>
<td>Artificial cerebrospinal fluid</td>
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<td>Ang-II</td>
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<td>AT1R</td>
<td>Angiotensin-II type 1 receptor</td>
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<td>BP</td>
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<td>NT</td>
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<td>Paraventricular nucleus</td>
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<td>RAS</td>
<td>Renin angiotensin system</td>
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<td>SA</td>
<td>Synapsin-hACE2</td>
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<tr>
<td>SBRS</td>
<td>Spontaneous baroreceptor reflex sensitivity</td>
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<td>SHR</td>
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INTRODUCTION

The brain Renin Angiotensin System (RAS) plays a critical role in the regulation of blood pressure (BP). Angiotensin-II (Ang-II), the main peptide in this system, by acting on brain Ang-II type 1 receptors (AT1R), can increase sympathetic outflow, BP, water intake, salt appetite, vasopressin release but also decreases baroreflex gain and vagal tone. Over-activity 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.

Angiotensin Converting Enzyme type 2 (ACE2) was discovered a decade ago and has been recognized as a critical component of the RAS. We previously identified the presence of ACE2 in the mouse brain, notably in regions involved in the control of cardiovascular function, supporting a potential role for ACE2 in central BP regulation. Indeed, by transforming Ang-II into the vasodilatory peptide Ang-(1-7), ACE2 not only reduces AT1R stimulation by Ang-II, but also promotes Mas receptors (MasR) activation by Ang-(1-7), leading to nitric oxide release and opposing the development of neurogenic hypertension.

In order to study the impact of the ACE2/Ang-(1-7)/MasR axis of the RAS in hypertension, over-expression of ACE2 has been used extensively in animal models. Studies from various groups, including ours, reported that ACE2 over-expression leads to a reduction in sympathetic drive, an improvement in baroreflex gain and overall, a reduction of BP in experimental hypertension models. However, fewer studies addressed the effects of RAS over-activation on endogenous ACE2 in neurogenic hypertension. In spontaneously hypertensive rats (SHR), ACE2 protein expression was shown to be reduced in the rostral ventrolateral medulla, a region containing pre-sympathetic neurons responsible for the maintenance of hypertension. Similarly, we previously observed reduced ACE2 activity in the brainstem of genetically hypertensive mice with chronic elevation of Ang-II. These findings suggest that while 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 DOCA-salt hypertensive mice, promoting the reduction of membrane-bound ACE2 in these animals. In addition, using transgenic mice, we show that maintaining high level of ACE2 specifically in the brain restores baroreflex and autonomic functions and contributes to the reduction of hypertension and associated inflammation. Finally, we demonstrated that RAS over-activity activates ADAM17 expression and activity in 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 non-transgenic (NT) littermates. 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.
Baseline BP was recorded by telemetry in uni-nephrectomized 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 and 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. BP was recorded daily by radiotelemetry as described above. Another subset of NT mice was infused with Losartan (2 µg/hour icv) for 3 weeks while receiving DOCA-salt treatment. Spontaneous baroreceptor reflex sensitivity (SBRS), reflecting the baroreflex control of heart rate (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 various assays.

Statistics.
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 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 (synapsin-hACE2; 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
ACE2 expression in neurons plays a critical role in maintaining the functionality of baroreflex and autonomic regulations during the development of hypertension.

ACE2 overexpression prevents DOCA-salt-mediated RAS over-activity.

ACE2 has been recognized as a member of the RAS, as 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 (NE) and vasopressin (AVP) release, thereby contributing to the maintenance of hypertension. We tested whether ACE2 expression could regulate the release of these pro-hypertensive neuropeptides by modulating Ang-II levels in the brain. Measurement of Ang-II levels using ELISA in NT+DOCA mice, revealed a 3-fold increase in the hypothalamus (P<0.05 vs. NT+sham, Figure 1F) but not in the plasma (NT+sham: 23 ±1 and NT+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 vs. NT+sham, Figure 1G) and urinary NE (P<0.05 vs. NT+sham, Figure 1H) levels were significantly increased following DOCA-salt treatment. ACE2 over-expression 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 vs. NT+DOCA, Figure 1F). Moreover, it was associated with a 50% reduction of AVP levels (Figure 1G, P<0.05 vs. NT+DOCA, P<0.05 for interaction between DOCA treatment and genotype) and prevented the rise in urinary NE levels (P<0.05 vs. NT+DOCA, Figure 1H).

While elevation of brain Ang-II levels has been reported to mediate an inflammatory response preceding 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 TNF-α, IL-1β, IL-6 and MCP-1 in the brain during DOCA-salt hypertension. Real-time RT-PCR shows that mRNA for all pro-inflammatory cytokines and chemokine were elevated in the hypothalamic paraventricular nucleus (PVN) of NT+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+DOCA mice, supporting the beneficial effects of ACE2 expression in the prevention of RAS-mediated inflammation.

Since RAS over-activity is associated with increased levels of classic RAS components such as Ang-II and the AT1R, as well as reduction of components of the compensatory RAS like 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 qRT-PCR and Western blotting. In NT mice, DOCA-salt treatment resulted in up-regulation of AT1R and a concomitant reduction of MasR levels in the PVN (Figure 3A-D), contributing to RAS over-activity. Conversely, ACE2 over-expression was associated with MasR up-regulation and prevented the receptors 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 the 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 over-activity, increase in pro-hypertensive peptides and pro-inflammatory 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 over-activity.

ADAM17 stimulates ACE2 shedding in the brain of DOCA-salt hypertensive mice.

The findings presented above suggest that ACE2 expression can exert an inhibitory role on RAS over-activity thus abating the development of hypertension. However, we and others previously showed that RAS over-activity is associated with down-regulation 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+DOCA mice, ACE2 activity was reduced by 50% in the hypothalamus (P<0.05 vs. NT+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. While 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 cerebrospinal fluid (CSF). Surprisingly, NT+DOCA mice exhibited a ~2-fold increase in ACE2 activity in the CSF (P<0.05 vs. NT+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+DOCA mice by the observation of increased ADAM17 activity and expression in the hypothalamus (P<0.05 vs. NT+sham, Figure 4D,E). To confirm that increased ADAM17 is mediated by RAS overactivity, protein expression was determined in losartan-treated hypothalami. Indeed, Online Figure III shows that blockade of AT1R 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, we then assessed CaM-bound ACE2 levels following immuno-precipitation. Interestingly, we observed a significant reduction of CaM-bound ACE2 in DOCA-salt-treated NT mice (P<0.05 vs. NT+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 over-activity-mediated mechanism leading to enhanced ADAM17 expression.
and activity that participates in ACE2 down-regulation 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. Indeed, it appears that ACE2 over-expression can limit the impact of ADAM17-mediated shedding and overcome the feed-forward mechanism of RAS over-activity.

**Knockdown of ADAM17 attenuates DOCA-salt-induced hypertension.**

The above findings suggest that RAS over-activity leads to up-regulation 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 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 nM, 24 hr) showed a significant increase in ADAM17 expression, confirming the ability of Ang-II to up-regulate this sheddase (Online Figure IV). However, pre-treatment with ADAM17 siRNA, but not scrambled siRNA, blocked ~90% of ADAM17 expression. This validation being achieved, we infused ADAM17 siRNA centrally (0.1 nmoles/day icv) in NT mice during DOCA-salt administration. While a 3-week icv infusion of ADAM17 siRNA had no effect on MAP or SBRS levels in sham-treated mice (P>0.05 vs. NT+sham, Figure 5A,B). Interestingly, knockdown of ADAM17 in the brain significantly attenuated DOCA-salt-induced hypertension (P<0.05 vs. NT+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+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 to the increase in ADAM17 expression in NT+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 ~50% knockdown of ADAM17 expression in the hypothalamus (Figure 5C) that 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, many reports have supported the pivotal role of this enzyme in cardiovascular diseases. However, while overexpression studies have established the benefits of ACE2, very little has been done 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 enzyme’s compensatory activity. Finally, we assessed whether prevention of ACE2 shedding could affect the development of DOCA-salt hypertension. 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 Ang-II-mediated up-regulation 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 suffering from high BP. Previous studies have demonstrated that DOCA-salt hypertension, a low-renin hypertension model, is mediated through activation of the RAS within the brain and is associated with enhanced sympathetic activity and vasopressin secretion. One of the many brain regions involved in the central regulation of BP is the hypothalamic PVN which contains both pre-sympathetic and vasopressinergic neurons. In addition, we previously reported that ACE2 is present in this region and capable of modulating cardiovascular function. In our study, while there was no alteration in plasma Ang-II levels, we observed over-activity 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, is detrimental for the compensatory RAS in the brain, leading to down-regulation 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. While, 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 indeed mediated by Ang-(1-7) via its MasR.

Previous studies have supported the interaction between centrally acting Ang-II and the systemic release of AVP. DOCA-salt treatment increases the release of AVP, which in turn strongly contributes to the pressor effect of centrally acting Ang-II. Interestingly, Ang-(1-7), the product of Ang-II degradation by ACE2 was originally reported to promote AVP release from hypothalamic explants. However, our in vivo studies argue against this hypothesis. Indeed, 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, was associated with a reduction of AVP release and 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 activation of pro-inflammatory mechanisms contribute 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 in which the central pressor effects of Ang-II lead to activation of T cells, which in turn, promote vascular inflammation and further raise BP, leading to severe hypertension. Despite previous reports showing increased pro-inflammatory 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 indeed associated with elevated pro-inflammatory 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. Our data show that DOCA-salt hypertension was associated with increased ADAM17 expression and activity in the hypothalamus, while membrane ACE2

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expression was reduced and soluble ACE2 increased in the CSF. Moreover, we observed that treatment of neurons with Ang-II resulted in up-regulation of ADAM17, consistent with previous reports showing that EGF receptor transactivation is mediated by Ang-II stimulation of ADAM17.37-39 The fact that ACE2 shedding could be reduced by ACE2 overexpression suggests that Ang-II is indeed 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 post-translational 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,40, 41 which upon activation by reactive oxygen species, is translocated to the plasma membrane where it can exert its shedding process. While ADAM17, also called TNFα convertase, can promote the shedding of multiple membrane proteins, including TNFα, its receptor and the EGF receptor,42 it is the only sheddase known to cleave ACE2, although the exact site of cleavage remains to be determined.43, 44 ADAM17-mediated ACE2 shedding has previously been reported in cell cultures,12, 45, 46 where cleavage of the membrane-bound ACE2 releases a soluble form that retains its carboxypeptidase activity.47 In addition, ACE2 shedding was recently suggested as a biomarker for chronic kidney disease.48 ADAM17 has been suggested as a player in vascular remodeling, hypertrophy and growth processes37, 38, 49, 50, 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 since it would allow for the reduction of active TNFα levels, thus reducing part of the inflammatory component and prevent ACE2 shedding, thereby preserving the compensatory function of this carboxypeptidase.

Interestingly, our study also identified weaknesses among the mechanisms supposed to preserve ACE2 function from shedding. CaM was previously reported to bind the cytoplasmic tail of ACE2 and thereby confer protection against ADAM17-mediated shedding.20 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 over-activity 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 proteins 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 up-regulation 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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REFERENCES


DISCLOSURES
None.


FIGURE LEGENDS

**Figure 1**: Neuron-targeted ACE2 over-expression attenuates DOCA-salt hypertension. Deoxycorticosterone acetate (DOCA) implanted subcutaneously and combined with 1% saline drinking solution induced a progressive rise of mean arterial pressure (MAP, A) in uni-nephrectomized (n=12 mice/group) non-transgenic (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) following ip 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 Ang-II (F), plasma AVP (G) and urinary norepinephrine (H) were determined using ELISA kits (n=3-6 mice/group). *P<0.05 vs. sham and †P<0.05 vs. NT+DOCA.

**Figure 2**: ACE2 overexpression reduces DOCA-salt induced inflammation. Quantitative real time RT-PCR measurements of pro-inflammatory cytokines (IL-1β, IL-6, and TNF) and chemokine (MCP-1) in hypothalamic paraventricular nucleus 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.

**Figure 3**: Neuron-specific ACE2 over-expression reverses the DOCA-salt induced changes in angiotensin receptors. Quantitative real time RT-PCR measurements of mRNA expression for AT1R (A) and MasR (B) in the hypothalamic paraventricular nucleus (PVN) of DOCA-salt-, or sham-, treated non-transgenic (NT) and syn-hACE2 (SA) mice. Representative western blots and densitometric analysis of group data for AT1R (C) and MasR (D) protein expression in the PVN. Data are means ±SEM (n=6 mice/group). *P<0.05 vs. sham and †P<0.05 vs. NT+DOCA.

**Figure 4**: ADAM17-mediated shedding impairs ACE2 expression and activity in DOCA-salt hypertension. (A) ACE2 activity assay from hypothalami isolated after 3 weeks of DOCA-salt or sham treatment in NT and SA mice (n=5-7/group). (B) Representative Western blot and quantitative data for mACE2 in 2 independent hypothalamus homogenates per group. (n=6 mice/treatment group). (C) ACE2 activity assay from microdialyzed CSF samples (n=4-6/group). (D) ADAM17 activity assay from hypothalami isolated after 3 weeks of DOCA-salt or sham treatment in NT and SA mice (n=5-7/group). (E) Representative Western blot showing both pro (upper band) and mature (lower band) forms and quantitative data for ADAM17 (n=6 mice/treatment group). (F) Representative immunoblotting for mACE2 following immunoprecipitation of calmodulin in 3 independent hypothalamic homogenates. (n=6 mice/treatment group). *P<0.05 vs. NT+sham and †P<0.05 vs. NT+DOCA. Abbreviations: AFU, arbitrary fluorescence units; AU, arbitrary units; mACE2, mouse ACE2.

**Figure 5**: Brain ADAM17 knockdown reduces BP and restores baroreflex sensitivity in DOCA-salt hypertension. MAP recording (A) and changes in SBRS (B) after 3 weeks of DOCA-salt or sham treatment in NT mice compared to baseline recordings obtained before treatment (n=5-7/group). (C) Representative Western blot and quantitative data for ADAM17 in hypothalamus homogenates. (n=3-6 mice/group). (D) ACE2 activity assay in the hypothalamus and CSF of NT mice following DOCA-salt or sham treatment in the presence or absence of ADAM17 siRNA (n=4/group). *P<0.05 vs. NT+sham and †P<0.05 vs. NT+DOCA.

**Figure 6**: Brain RAS, ADAM17 and neurogenic hypertension. On baseline, Angiotensin(Ang)-II is formed from Ang-I by the angiotensin converting enzyme (ACE) and can bind 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 DOCA-salt stimulation results in increased levels of Ang-
II and AT$_1$R 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 up-regulation.
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 DOCA-salt–induced hypertensive mice.
- ACE2 overexpression in the brain attenuates DOCA-salt-induced hypertension by reversing changes in AT1R and MasR 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, 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 angiotensin II levels during DOCA-salt–induced hypertension results in up-regulation 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. While our investigation focuses on the brain renin-angiotensin system, we speculate that the findings might be relevant to diseases featuring over-activity of the renin-angiotensin system.
Brain ACE2 Shedding Contributes to the Development of Neurogenic Hypertension
Huijing Xia, Srinivas Sriramula, Kavaljit H Chhabra and Eric Lazartigues

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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\(^1\) 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 \textit{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.\(^2\) 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)\(^3\) 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.\(^1\), \(^2\) 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 (\(\beta\)-blocker, 4 mg/kg), atropine (muscarinic receptor blocker, 1 mg/kg) and chlorisondamine (ganglionic blocker, 5 mg/kg).\(^4\) Each injection was separated by at least a 3-hour recovery period. Changes in HR (\(\Delta HR\)) or mean arterial pressure (\(\Delta MAP\)) 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\(^{\circ}\)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,\(^1\), \(^2\) using rabbit anti-mACE2 (Santa Cruz, sc-1:500), rabbit anti-ADAM17 (Abcam, ab2051; 1:1000), goat anti-AT\(_1\)R (Santa Cruz, sc-9040; 1:1000) and goat anti-MasR (Alomone, 1:500) antibodies. Equal loading was determined using either \(\alpha\)-actinin, \(\alpha\)-tubulin or \(\gamma\)-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 (2×10⁵ 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 hypothalamus 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 ∆∆Cₜ 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 CaCl₂: 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

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<tr>
<th>Gene</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
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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.