α-LIPOIC ACID REDUCES NEUROGENIC HYPERTENSION BY BLUNTING OXIDATIVE STRESS-MEDIATED INCREASE IN ADAM17

Thyago M. de Queiroz, Huijing Xia, Catalin M. Filipeanu*, Valdir A. Braga and Eric Lazartigues

Department of Pharmacology and Experimental Therapeutics (T.M.Q., H.X., A.C., C.M.F., E.L.), Neurosciences (E.L.) and Cardiovascular Centers of Excellence (H.X., C.M.F., E.L.), Louisiana State University Health Sciences Center, New Orleans, LA; and Department of Biotechnology (T.M.Q., V.A.B.) – Federal University of Paraíba, João Pessoa, PB – Brazil.

*Current address: Department of Pharmacology, Howard University College of Medicine, Washington, DC 20059

Running title: ADAM17 and Oxidative Stress in Hypertension

Correspondence to Eric Lazartigues, Louisiana State University Health Sciences Center, School of Medicine, Department of Pharmacology and Experimental Therapeutics, 1901 Perdido St, Rm 5218, New Orleans, LA 70112. E-mail: elazar@lsuhsc.edu

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Abstract

We previously reported that ACE2 compensatory activity is impaired by the disintegrin and metalloprotease 17 (ADAM17) and lack of ACE2 is associated with oxidative stress in neurogenic hypertension. To investigate the relationship between ADAM17 and oxidative stress, Neuro2A cells were treated with Ang-II (100 nM) 24 h after vehicle or α-lipoic acid (LA: 500 µM). ADAM17 expression was increased by Ang-II (120.5 ±9.1 vs. 100.2 ±0.8%, P<0.05) and decreased after LA (69.0 ±0.3 vs. 120.5 ±9.1%, P<0.05). In other set of experiments, LA reduced ADAM17 (92.9 ±5.3 vs. 100.0 ±11.2%, P<0.05) following its overexpression. Moreover, ADAM17 activity was reduced by LA in ADAM17-overexpressing cells (109.5 ±19.8 vs. 158.0 ±20.0 FU/min/µg protein, P<0.05), in which ADAM17 overexpression increased oxidative stress (114.1 ±2.5 vs. 101.0 ±1.0%, P<0.05). Conversely, LA-treated cells attenuated ADAM17 overexpression-induced oxidative stress (76.0 ±9.1 vs. 114.1 ±2.5%, P<0.05).

In DOCA-salt-hypertensive mice, a model in which ADAM17 expression and activity are increased, hypertension was blunted by pre-treatment with LA (119.0 ±2.4 vs. 131.4 ±2.2 mmHg, P<0.05). In addition, LA improved dysautonomia and baroreflex sensitivity. Furthermore, LA blunted the increase in NADPH oxidase subunits expression, as well as the increase in ADAM17 and decrease in ACE2 activity in the hypothalamus of DOCA-salt hypertensive mice.

Taken together, these data suggest that LA might preserve ACE2 compensatory activity by breaking the feed-forward cycle between ADAM17 and oxidative stress, resulting in a reduction of neurogenic hypertension.

Key Words: ADAM17, oxidative stress, DOCA-salt hypertension, antioxidant, ACE2
Novelty
This study highlights the relationship between ACE2 and ADAM17 in hypertension emphasizing the role of oxidative stress as a mediator between the AT1 receptor and ADAM17 in the mouse brain. Furthermore, our study shows the benefits of lipoic acid treatment in preventing ADAM17 up-regulation and thus the development of hypertension.

Introduction
The renin-angiotensin system (RAS) is an important regulator of arterial blood pressure (BP). Angiotensin-II (Ang-II) is the major effector molecule of the RAS, which exerts its biological effects mainly via the type-1 Ang-II receptor (AT1R). Therefore, Ang-II acts as a potent vasoconstrictor, regulates water intake and salt metabolism and increases sympathetic outflow and BP (27).

Abundant evidence suggests that a key mechanism by which Ang-II contributes to hypertension is increasing oxidative stress (48). Oxidative stress is a condition of increased production of reactive oxygen species (ROS) and/or reduction of scavenging mechanisms (38). Previous studies from our laboratory and others have shown that Ang-II administration into the central nervous system, as well as in the periphery, induces hypertension via NAD(P)H oxidase (Nox)-mediated ROS production in different cardiovascular regulatory brain regions (38, 48). Therefore, ROS are important mediators in Ang-II-dependent hypertension.

A new important component of the RAS is the type-2 angiotensin converting enzyme (ACE2). ACE2, a homolog of ACE, can cleave Ang-I and Ang-II to Ang-(1-9) and Ang-(1-7), respectively (43). Ang-(1-7), the product of Ang-II
degradation by ACE2, has opposite properties to that of Ang-II, by acting on the Mas receptor (MasR). The ACE2/Ang-(1-7)/MasR axis of the RAS promotes vasodilation, anti-proliferation and reduction of heart failure (39).

ACE2 is a membrane protein which can undergo shedding to release a catalytically active ectodomain from the cell surface into the extracellular milieu (23). The proteases involved in this process are called sherdases and they control the biological activity of membrane proteins (10). A well-known sherdase is a disinteigrin and metalloproteinase 17 (ADAM17), also called tumor necrosis factor-(TNF-α)-converting enzyme (1).

Studies have shown that Ang-II induces ADAM17-mediated transactivation of the epidermal growth factor receptor (EGFR) and hypertrophy of vascular smooth muscle cells (26). Other in vitro studies demonstrated that ROS are also able to activate ADAM17 in platelets and this effect could characterize a limiting mechanism for platelet function (6). Furthermore, Nox4-mediated ROS production is required to increase ADAM17 expression and subsequent induction of cardiac hypertrophy (46).

Therefore, we hypothesized that chronic RAS activation enhances oxidative stress and ADAM17 activity thus promoting ACE2 shedding. To test this hypothesis, we used an antioxidant, α-lipoic acid (LA), in order to decrease oxidative stress and down-regulate ADAM17 expression. LA, also known as 1,2-dithiolane-3-pentanoic acid or thiocotic acid, is a disulfide antioxidant and exists in both R- and S-enantiomeric forms. However, only (R)-LA is conjugated to conserved lysine residues in an amide linkage, thus making this isoform essential as a cofactor in biological systems (31). We recently documented that LA reduces BP and improves baroreflex sensitivity in renovascular hypertensive
rats (29). However, the mechanisms underlying those effects remain unknown.

To address this, we used Neuro2A cells (neuroblastoma cell line) and an experimental model of neurogenic hypertension combining chronic administration of deoxycorticosterone acetate (DOCA), reduced renal mass and a high salt diet (DOCA-salt model) (37).

Material and Methods

Animals
Experiments were performed in male C57BL/6J mice (6 to 9 weeks of age; 25 to 30 g) (Jackson laboratories). Animals were housed in a temperature- and humidity-controlled facility under a 12 h 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. BP measurements using radio-telemetry and DOCA-salt treatments in mice were performed as previously described (37).

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).

Baseline BP was recorded by telemetry in uni-nephrectomized mice for 3 days, then mice were randomly divided into 4 groups (n=6/group), all 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 for DOCA-implanted mice was replaced by 1% NaCl solution. From the day of DOCA implantation, mice were treated with tempol (258 mg/kg/day) or LA (600 mg/kg/day) for 21 days orally. Drugs were mixed with a banana-flavored gel pudding (LabGel®, ClearH2O) to form dispensable pellets according to body weight. Gel pellets were provided daily with the chow to the following groups: Sham (n=6); DOCA (n=6); DOCA + TEMPOL (n=6) and DOCA + LA (n=6). BP was continuously recorded for 3 additional weeks. Spontaneous baroreceptor reflex sensitivity (SBRS), reflecting the baroreflex control of heart rate, was calculated using the sequence method, as previously described (14). Autonomic function was assessed in conscious freely moving mice before and 3 weeks after DOCA-salt and antioxidant treatment, using a pharmacological method involving ip injections of propranolol (β-blocker, 4 mg/kg), atropine (muscarinic receptor blocker, 1 mg/kg) and chlorisondamine (ganglionic blocker, 5 mg/kg) (38) (36). Each injection was separated by a 3-hour recovery period. Maximum changes in heart rate (ΔHR) or mean arterial pressure (ΔMAP) were calculated following administration of these blockers. At the end of the protocol, mice were euthanized and the brain was collected and stored at -80 °C until used in the biochemical assays.

Cell Culture

Neuro2A mouse neuroblastoma cells (ATCC Manassas, VA) were grown in minimum essential medium (MEM; GibCO, Invitrogen, Carlsbad, CA) with L-glutamine (2 mM) and Eagle’s balanced salt solution adjusted to contain sodium
bicarbonate (1.5 g/L), non-essential amino acids (0.1 mM), sodium pyruvate (1.0 mM) and fetal bovine serum (10%; GIBCO) at 37 °C under a humidified 95% and 5% (v/v) mixture of air and CO₂. Neuro2A cells were pretreated with either tempol (10 µM in PBS) or LA (500 µM in PBS + 0.03% NaOH) and 24 hours later treated with Ang-II (100 nmol/L in PBS) for 24 hours. Cells were grown onto chamber slides at a density of 1 x 10⁵ cells/chamber for dihydroethidium (DHE) fluorescence measurement or 10 cm dishes at a density of 10⁶ cells/dish for western blot, ADAM17 assay and QRT-PCR experiments.

For experiments using ADAM17 overexpression, cells were treated with mADAM17 in pcDNA3.1 plasmid (1.25 µg/ml, from Addgene, Cambridge – MA, cat. no. 19141) and GFP plasmid (1.25 µg/ml, pEGFP-C3, Clontech, cat. no. 6082-1) was used as a control of successful transfection. Both plasmids are cytomegalovirus promoter driven. For both plasmids we used Lipofectamine transfection reagents (Invitrogen - Life Technologies). After 24 hours, LA (500 µM in PBS + 0.03% NaOH) was added into the cells for 24 hours in serum-free medium. Cells were then harvested for western blotting analysis, DHE staining, ADAM17 activity assay and QRT-PCR, as described above.

**Western blot**

Cell pellets were incubated in 300 µL lysis buffer (in mmol/L: HEPES: 10, NaCl: 150, MgCl₂: 5, EGTA: 1, 0.02% (w/v) NaN₃, pH 7.4) containing a protease inhibitors cocktail (Sigma, St Louis, MO). Protein concentration was measured using a BCA assay kit (Pierce, Rockford, IL). Cell lysates were mixed with SDS-PAGE sample buffer, heated and loaded onto a 4–15% SDS–polyacrylamide gel for electrophoresis (Life Technologies, Carlsbad, CA). Proteins were
transferred to nitrocellulose membrane (Fisher Scientific, Houston, TX). Membranes were blocked with 5% non-fat milk in PBST (1.47 mM NaH₂PO₄, 8.09 mM Na₂HPO₄, 145 mM NaCl, 0.05% v/v Tween-20H, 0.01% w/v thimerosal, pH 7.4) for 1 hr at room temperature and incubated with anti-ADAM17 (Abcam, ab2051, 1:1000), at 4 ºC, overnight. Membranes were washed with PBST 3 times for 5 min then incubated with goat anti-rabbit IgG-HRP (Perkin Elmer; 1:5000) for 1 hour at room temperature. Specific bands were detected by chemiluminescence according to the manufacturer’s instructions (ECLH, Perkin Elmer) at room temperature and re-probed with mouse anti-γ-tubulin (Sigma, #5192; 1:10000) and goat anti-mouse IgG-HRP. Bands corresponding to specific antibodies were quantified by densitometry (ImageJ version 1.45 K) and normalized to γ-tubulin. Proteins extracted from hypothalamus (20 µg) were processed for Western blotting as previously described (14). Briefly, after euthanasia, brains were quickly removed from the animals and immediately frozen on dry ice. Total protein was extracted from hypothalamic tissue and equal amounts were loaded and separated by SDS-PAGE and electrophoretically transferred to the membranes. The membranes were washed and incubated with following primary antibodies: anti-ADAM17 (Abcam, ab2051, 1:1000); anti-Nox4 (Abcam, ab109225, 1:1000) and mouse anti-Nox2 (BD Transduction Laboratories®, cat. no. 611415, 1:2000).

**Fluorogenic monitoring of superoxide production**

Neuro2A cells were incubated in serum-free medium in the presence of ADAM17 and GFP plasmids for 24 hours. On the next day post-transfection,
GFP expression was observed using a fluorescence microscope (Olympus, IX81; Excitation/emission wavelengths: 488/509 nm) as an index of successful transfection and cells were treated for 24 h with LA (500 µM). The oxidant-sensitive fluorogenic probe DHE (2 µmol/L) was loaded for 30 minutes. Cells were washed thrice in the dark with PBS and examined on a fluorescence microscope (Olympus U-TB190, Japan; Excitation/Emission wavelength: 518/605 nm). Each experiment was performed in triplicate. DHE fluorescence was quantified using Image J software.

ADAM17 activity assay

ADAM17 activity was measured in Neuro2A cells or hypothalamus (6 µg proteins/well) using a TACE activity kit (Sensolyte 520 TACE activity assay, ANASPEC), following the manufacturer’s instructions. Fluorescence emission was monitored using a SpectraMax M2 Fluorescence Reader (Excitation/emission wavelengths: 490/520 nm Molecular Devices, Sunnyvale, CA) during 2 hours. Data are presented as amount of enzymatic reaction normalized for total protein and expressed as arbitrary fluorescence units (AFU).

ACE2 activity assay

ACE2 activity was measured as previously described (28). Tissue from brain hypothalamus was homogenized with ACE2 activity reaction buffer, centrifuged and the supernatant transferred to a clean tube. ACE2 activity measurement was carried out in the presence of captopril to eliminate any contribution by endogenous ACE and based on the use of the Fluorogenic Peptide Substrate

Non-specific enzyme activity was measured by including DX600 (1 μmol/L), a specific ACE2 inhibitor, (Phoenix Pharmaceutical, Belmont, CA). Fluorescence emission was monitored using a SpectraMax M2 Fluorescence Reader (Molecular Devices, Sunnyvale, CA). Data are presented as amounts of substrate FPSVI converted to product per minute, normalized for total protein and expressed as AFU.

**Measurement of mRNA expression (QRT-PCR)**

Total RNA was isolated from Neuro2A cells using RNeasy Mini Kit (Qiagen, cat. no. 74104) and then we performed the DNase digestion using RNeasy-free DNase set (Qiagen, cat no. 79254). 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). Primer sequences used for mADAM17 (Integrated DNA Technologies – IDT) were (554F) Forward: 5′-CGT GGT TGG TGA GCC TGA CT-3′ and (643R) Reverse: 5′-TTA TAT TCT GCC CCA TTG TGT TTG-3′. Data were analyzed by the ∆∆CT comparative method and expressed as fold changes compared to control group.

**Statistical Analysis**

Data are expressed as mean ±SEM. Data were analyzed by Student t test or one-way ANOVA, followed by Newman–Keuls correction for multiple comparisons between means as appropriate. Statistical comparisons were
performed using Prism 5 (GraphPad Software, San Diego, CA). Difference was considered significant when $P < 0.05$. 
Results

LA prevents the increase in Ang-II-induced ADAM17 expression in Neuro2A cells

We previously reported that ADAM17 expression is increased after Ang-II treatment in Neuro2A cells (37). Here, we investigated whether oxidative stress is involved in ADAM17 modulation. Neuro2A cells incubated with Ang-II (100 nmol/L, 24 hours) exhibited an increase in ADAM17 protein expression compared to control (120.5 ±9.1 vs. 100.2 ±0.8%, p<0.05, Figure 1A). This increase was reduced by pretreatment with either LA (69.0 ±0.3 vs. 120.5 ±9.1%, P<0.05, Figure 1A) or tempol (87.0 ±0.7 vs. 118.4 ±7.0%, P<0.05, Figure 1B). These data confirm the Ang-II-mediated up-regulation of neuronal ADAM17 and suggest a critical role for oxidative stress in this process.

LA reduces ADAM17 overexpression and activity in Neuro2A cells

In order to clarify the mechanisms involved in LA-mediated reduction of ADAM17 expression, cells were transfected with a plasmid overexpressing ADAM17. Western Blotting analysis revealed that transfection with the ADAM17 plasmid lead to ~100% increase in the sheddase’s expression in Neuro2A cells compared to control (239.3 ±27.6 vs. 100.0 ±16.5%, P<0.05, Figure 2A). ADAM17 overexpression tends to reduce after 24 hours of LA treatment (239.3 ±27.6 vs.195.0 ±25.6%, Figure 2A).

In order to investigate a possible effect of LA on ADAM17 transcriptional regulation, we first measured mRNA levels for ADAM17. As expected, ADAM17 overexpression significantly increased mRNA expression for ADAM17 when compared to control (234.3 ±10.1 vs. 1.7 ±0.1 fold, P<0.05, Figure 2B).
However, LA was unable to affect the increase in ADAM17 mRNA expression following transfection with the ADAM17 plasmid in Neuro2A cells, as shown in Figure 2B. These data rule out a potential effect of LA on ADAM17 transcriptional regulation, suggesting that LA directly acts on ADAM17 protein expression and/or activation.

To determine whether these changes are restricted to ADAM17 protein expression, ADAM17 activity was assessed. Neuro2A cells transfected with the ADAM17 plasmid showed a ~2-fold increase in ADAM17 activity (158.0 ±20.0 vs. 77.3 ±3.3 FU/min/µg protein, p<0.05, Figure 2C). LA reduced ADAM17 activity in Neuro2A (109.5 ±19.8 vs. 158.0 ±20.0 FU/min/µg protein, p<0.05, Figure 2C).

LA prevents ADAM17-mediated oxidative stress

To further investigate whether ADAM17 overexpression could be involved in oxidative stress and whether LA decreases ADAM17 expression and activity by its antioxidant effect, cells were transfected with ADAM17 plasmid and treated with LA. Oxidative stress was assessed by DHE staining. ADAM17 overexpression significantly increased superoxide levels in Neuro2A cells (114.1 ±2.5 vs. 101.0 ±1.0%, p<0.05, Figure 2D), while LA treatment attenuated the ADAM17-induced oxidative stress (76.0 ±9.1 vs. 114.1 ±2.5%, p<0.05, Figure 2D). Taken together, these data support the beneficial effect of LA in preventing ADAM17-mediated ROS formation in neurons.
LA prevents DOCA-salt-induced hypertension by improving baroreflex sensitivity and autonomic function

DOCA-salt treatment elicited a rapid rise in MAP within 48 hours (119.4 ±2.7 mmHg vs 102.5 ±2.4 mmHg, p<0.05, n=7, Figure 3A) that reached a plateau in the 2nd week compared to Sham group (131.4 ±2.2 vs. 100.2 ±2.9 mmHg, p<0.05, n=7, Figure 3A). Importantly, LA and tempol produced a similar abatement of hypertension (Figure 3A), as observed in DOCA + LA (119.0 ±2.4 vs. 131.4 ±2.2 mmHg, p<0.05 n=7) and DOCA + tempol (111.0 ±8.7 vs. 131.4 ±2.2 mmHg, p<0.05 n=7) groups, confirming that oxidative stress is a critical contributor in this model.

Cardiac and vascular sympathetic drive were increased after DOCA-salt treatment compared to Sham (p<0.05, Figure 3B,D), while the vagal tone was blunted in DOCA-salt hypertensive mice (p<0.05, Figure 3C). Tempol and LA similarly reduced dysautonomia (i.e. reduced sympathetic drive and enhanced vagal tone) although LA exhibited a more modest effect on vascular sympathetic drive, with only a trend to reduce vagal tone. To determine the role of antioxidant therapy in DOCA-salt hypertension, we assessed spontaneous baroreflex sensitivity (SBRS) using the sequence method (14, 36). As expected, SBRS was significantly impaired in DOCA-salt hypertensive mice in comparison with Sham (0.96 ±0.1 vs. 2.2 ±0.3 msec/mmHg, P<0.05, Figure 3E). However, both tempol and LA prevented the reduction of SBRS in DOCA-salt hypertensive mice (1.8 ±0.2 and 1.94 ±0.2, respectively vs. 0.96 ±0.1 msec/mmHg, P<0.05, Figure 3E). Altogether, these data suggest that LA and tempol reduce DOCA-salt hypertension by preventing autonomic dysfunction and normalizing baroreflex sensitivity.
LA reduces ACE2 shedding in DOCA-salt hypertension

We previously reported that ADAM17 promotes ACE2 shedding in DOCA-salt hypertension (37). Accordingly, ADAM17 activity was increased by 25% and ACE2 activity was reduced by 20% in the hypothalamus after 3 weeks of DOCA-salt treatment ($P<0.05$ vs. sham, Figure 4). Interestingly, ADAM17 activity was blunted by tempol ($366.1 \pm 27.0$ vs. $546.8 \pm 14.0$ FU/min/µg protein, $P<0.05$ Figure 4A) and LA ($445.0 \pm 0.9$ vs. $546.8 \pm 14.0$ FU/min/µg protein, $P<0.05$ Figure 4A). In addition, LA prevented the reduction in ACE2 activity ($97.8 \pm 4.5$ vs. $80.8 \pm 3.3$% FU/min/µg protein, $P<0.05$ Figure 4B). Our data suggest that the reduction of oxidative stress by LA restores ACE2 activity by preventing ADAM17-mediated shedding.

LA reduces the enhanced brain expression of Nox2 and Nox4 in DOCA-salt hypertension

Since Nox2 and Nox4 are members of the NADPH oxidase family of enzymes involved in ROS production in hypertension, we tested whether these enzymes were altered in DOCA-salt hypertension and whether their protein expression is affected by LA treatment. As shown in Figure 5, oxidative stress was increased in DOCA-salt hypertension with an increase in protein expression for Nox2 ($367.1 \pm 71.0$ vs. $100.0 \pm 21.0$%, $P<0.05$, Figure 5A,B) and Nox4 ($172.3 \pm 20.1$ vs. $100.0 \pm 12.1$%, $P<0.05$, Figure 5A,C). LA was more effective than tempol in preventing the increase in both members of the Nox family ($P<0.05$, Figure 5A-C).
Discussion

Using a combination of *in vitro* and *in vivo* approaches, we tested the hypothesis that oxidative stress-mediated activation of ADAM17 and the resulting ACE2 shedding are blunted by LA in neurogenic hypertension. The major findings of this study are that LA attenuates the DOCA-salt hypertension-mediated dysautonomia and impaired baroreflex sensitivity, leading to a reduction in high BP. In neuronal cells, LA reduced the increase in ADAM17 expression and activity. Finally, the beneficial effects of LA were associated with its antioxidant activity.

Our data show that Ang-II treatment in Neuro2A cells induces an increase in ADAM17 protein expression. Increase in ADAM17 expression has been reported in several models of cardiovascular diseases, including hypertension (25). Several mechanisms have been proposed by which Ang-II induces ADAM17 activation (24, 26, 46). An important factor involved in ADAM17 activation is through ROS production. Strong evidence suggests that ROS stimulates the removal of the inhibitory pro-domain of ADAMs (47), and this leads to activation of ectodomain shedding (30). ROS also could mediate the phosphorylation of p38 MAP kinase (MAPK), which in turn phosphorylates and activates the threonine-735 residue on ADAM17 protein (33, 42). However, N-acetyl-L-cysteine was shown to impair metalloprotease-dependent ectodomain shedding by preventing ROS-mediated ADAM17 activation (30). Our study extends previous findings since LA not only reduced ADAM17 overexpression but also blunted ADAM17 activity, suggesting an inhibitory response in ADAM17 expression/activity due to an antioxidant effect.
Ang-II induces ROS production, including superoxide (O$_2^-$), in neurons of cardiovascular regulatory brain areas such as the paraventricular nucleus of the hypothalamus, the subfornical organ and the rostral ventrolateral medulla (5, 7, 48), and decreases ACE2 expression in Neuro2A cells (38). These alterations could be both the cause and consequence of an increase in ADAM17 expression and/or activity. Therefore, we assessed whether ADAM17 overexpression could trigger ROS production and whether LA decreases the sheddase by its antioxidant effects. DHE staining showed strong evidence that LA induces ADAM17 impairment by reducing oxidative stress. Previous data have shown that adenovirus-mediated ACE2 overexpression reduces ROS formation (15). Furthermore, ACE2 depletion could lead to an increase in ROS production (38), therefore we identified ACE2 activation as a new “antioxidant” effect. From our knowledge, the current study provides the first demonstration that elevated ROS production induces ADAM17 activation in neurons.

DOCA-salt hypertension is a well-established model of hypertension, depending on high salt and water intake leading to hypervolemia (17). In this model, excess of mineralocorticoids leads to an imbalance in renal sodium handling by increasing sodium and water reabsorption (45). Notably, after chronic DOCA-salt treatment the peripheral RAS, a major regulator of BP (18), is suppressed, while in the brain, there is overactivity of this system (20, 21). Previous studies have supported the hypothesis that DOCA-salt hypertension is a result of ACE up-regulation in the brain (21) or increase in AT$_1$R density in some regions of the brain such as subfornical organ, nucleus tractus solitari, area prostrema and paraventricular nucleus (19). RAS overactivity associated with AT$_1$R increase in the brain led us to suggest oxidative stress as a key mechanism in Ang-Ill-
mediated neurogenic hypertension (7). Increased oxidative stress has been found in DOCA-salt hypertension (2, 32). Oxidative stress mediates increased transcription of genes, including activation of nuclear factor-kB (NF-kB), responsible for the early inflammatory response in mineralocorticoid hypertensive animals contributing to end-organ damage (32). In the current study, we demonstrated that DOCA-salt hypertensive mice presented a greater increase in MAP and that either LA or the classic antioxidant tempol attenuated the hypertension. Antioxidant therapies, including ROS scavengers and vitamins, SOD mimetics or NADPH oxidase inhibitors have been shown to attenuate or prevent the development of hypertension (4, 8, 29). Our previous study showed that LA reduced hypertension in 2K1C rats (29). Besides, LA was able to decrease systolic BP of DOCA-salt hypertensive rats at a dose considerably higher compared to the one used in our study (35). We previously demonstrated that spontaneous baroreflex sensitivity and sympathetic activity are impaired in DOCA-salt hypertension (37). In our study, LA treatment improved impaired cardiac sympathetic and vagal tone with only a trend to affect the vascular sympathetic drive. Previous studies have shown that tempol treatment induced a reduction in BP by inhibition of sympathetic nerve activity (SNA) in DOCA-salt hypertension (41). In fact, tempol administration produced a greater depressor effect in DOCA-salt hypertensive rats compared to control ones, indicating that SNA acts as an important mediator of high BP in DOCA-salt hypertension (40). Curiously, the depressor effect induced by tempol in DOCA-salt hypertension was oxidative stress-independent (40), suggesting a direct effect of tempol on SNA without affect ROS-induced mechanisms in this model. On the other hand the effect observed for LA seems to be different from
that induced by tempol. In prior studies and in the present work, we showed that LA reduces the SNA and BP by an oxidative stress-dependent mechanism (4, 29). In addition, LA increased SBRS in DOCA-salt hypertensive animals. Moreover, our group previously showed that improvement of autonomic function is associated with ACE2-mediated reduction of oxidative stress in the central nervous system (38). Other work demonstrated that ACE2 gene therapy can restore baroreflex and autonomic functions and prevent the development of hypertension (14). Additionally, we documented that knockdown of ADAM17 prevents the reduction of brain ACE2 levels and inhibits DOCA-salt hypertension, suggesting that ACE2 shedding contributes to the development of neurogenic hypertension (37).

We next assessed the activity of ADAM17 and ACE2 in hypothalamus of DOCA-salt hypertensive mice. Activity of ADAM17 was augmented while ACE2 activity was decreased in DOCA-salt hypertensive animals. These responses were previously observed by us (37), however both activities were restored by antioxidant treatment with LA. A previous study showed that ACE2 overexpression in the PVN increased ACE2 activity and down-regulated AT1R expression contributing to the reduction of Ang-II-mediated hypertension (34). Ang-II mediates ADAM17 activation and ACE2 shedding through AT1R, acting as a feed-forward mechanism in the RAS. ACE2/Ang-(1-7)/MasR pathway is the main negative regulator of ACE/Ang-II/AT1R effects (43). Therefore, oxidative stress from Ang-II-mediated Nox activation can induce an up-regulation of ADAM17, which in turn induces ACE2-shedding and drives the feed-forward mechanism of hypertension. These responses corroborate the idea that both antioxidant therapy as well as an increase in ACE2 expression
counteract ROS formation in the brain and are pivotal to prevent neurogenic hypertension. The impaired ACE2 activity and ADAM17 activation found in pathological conditions, such as in DOCA-salt hypertension allow for the uncoupling of ACE2/Ang-(1-7)/MasR pathway and promote the increase of soluble ACE2 in cardiovascular diseases (9, 13).

A recent study reported that ADAM17 activation induces oxidative stress via up-regulation of Nox4 protein expression and increased Nox activity leading to extracellular matrix accumulation in kidney (16). In contrast, another study demonstrated that Nox4 and ROS promote increase in ADAM17 expression by Ang-II-mediated EGFR activation to induce cardiac hypertrophy (46). Another study suggested that Nox2 seems to be a more prominent mediator of the injurious effects of Ang-II in the central nervous system during hypertension (11), while injections of adenoviral vectors encoding siRNA targeting Nox2 or Nox4, to knockdown expression in the PVN, showed that both attenuated the development of aldosterone/NaCl-mediated hypertension (44). Of note, our study shows that both Nox2 and Nox4 are up-regulated in hypothalamus of DOCA-salt hypertension mice. In addition, LA treatment to avoid ROS formation blunted Nox subunits overexpression. This suggests that Nox has an important role in DOCA-salt hypertension development. Therefore ROS formation from Nox could induce ADAM17 overexpression and subsequently ACE2 shedding in the brain, thus promoting neurogenic hypertension.

Clearly, the mechanisms by which LA acts to promote the effects presented in this study need to be further investigated. However, we already know that LA regenerates endogenous antioxidants (vitamins C and E) and has the ability to scavenge free radicals (3). LA is widely known to reduce the expression of
metalloproteinase-9 through the inhibition of NF-kB (22), which is important to regulate an inflammatory response induced by ROS. In addition, one must keep in mind that ACE2 is only one of multiple targets for ADAM17 and therefore by reducing ADAM17 levels, LA could exert its beneficial effects via numerous other players, including EGFR, TNFα and various cytokines (12), ultimately inhibiting ROS formation and reducing hypertension.

In summary, our data suggest that LA might preserve ACE2 compensatory activity by breaking the feed-forward cycle between oxidative stress and ADAM17 due to its antioxidant properties. Therefore, ADAM17 could be a new target to prevent neurogenic hypertension.

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Disclosures
None.

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FIGURE LEGENDS

Figure 1. ADAM17 expression induced by Angiotensin-II in Neuro2A cells. Representative Western Blots, showing the pro- and mature forms of ADAM17 and group data (mature ADAM17), showing the reduction of ADAM17 expression induced by (A) lipoic acid (LA) or (B) Tempol. *P<0.05 vs. control and #P<0.05 vs. Ang-II. All data are means ±SEM.
Figure 2. Effects LA on ADAM17 overexpression induced by ADAM17 plasmid in Neuro2A cells. (A) Representative Western Blot and group data showing a trend to reduce the overexpression of ADAM17 induced by lipoic acid (LA). (B) QRT-PCR shows no change in ADAM17 expression after LA treatment. (C) ADAM17 activity is reduced by LA treatment. (D) Representative DHE-stained sections and group data showing that ADAM17 overexpression significantly increases ROS production in Neuro2A cells and that LA reduces ROS accumulation. GFP: Green fluorescent protein. *P<0.05 vs. Control, #P<0.05 vs. ADAM17. All data are means ±SEM.

Figure 3. Lipoic acid prevents DOCA-salt-induced hypertension. (A) Average mean arterial pressure changes in the various groups before and after DOCA-salt treatment. Lipoic acid improves impaired cardiac sympathetic (B) and vagal tone (C) in DOCA-salt-hypertension without affecting vascular sympathetic drive (D). Lipoic acid improves baroreflex sensitivity in DOCA-salt-induced hypertensive mice (E). *P<0.05 vs. Sham and #P<0.05 vs. DOCA. All data are means ±SEM.

Figure 4. ADAM17 and ACE2 activities in the hypothalamus of DOCA-salt hypertensive mice. The increase of hypothalamic ADAM17 activity (A) and the decrease of ACE2 activity (B) were reduced by lipoic acid treatment in DOCA-salt hypertensive mice. *P<0.05 vs. Sham and #P<0.05 vs. DOCA. All data are means ±SEM.
Figure 5. Hypothalamic NADPH oxidase expression in DOCA-salt hypertensive mice. Representative Western Blot and group data showing that lipoic acid reduces the increase in NOX2 (A) and Nox4 (B) protein expression in the hypothalamus of DOCA-salt hypertensive mice. *$P<0.05$ vs. Sham, #$P<0.05$ vs. DOCA and $^{&}P<0.05$ vs. DOCA + TEMPOL. All data are mean ±SEM.