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# O MECANISMO HIPOTENSOR DO ÁCIDO α-LIPÓICO EM MODELOS DE HIPERTENSÃO ARTERIAL DEPENDENTES DE ANGIOTENSINA II ENVOLVE A INIBIÇÃO DA ADAM17

**João Pessoa**
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TESE apresentada como requisito à obtenção do grau de doutor pelo curso de Pós-graduação em Produtos Naturais e Sintéticos Bioativos - Área de concentração: Farmacologia, do Centro de Ciências da Saúde, da Universidade Federal da Paraíba.

**Orientador: Prof. Dr. Valdir de Andrade Braga**

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**Thyago Moreira de Queiroz**

# Resumo

**O mecanismo hipotensor do ácido α-lipóico em modelos de hipertensão arterial dependentes de angiotensina II envolve a inibição da ADAM17.** QUEIROZ, T. M.; Tese de doutorado, Programa de pós-graduação em Produtos Naturais e Sintéticos Bioativos, PgPNSB/CCS/UFPB (2014)

## RESUMO

O estresse oxidativo tem sido apontado como um mecanismo fundamental na hipertensão arterial dependente de Ang II. Além disso, o estresse oxidativo está associado com a regulação negativa da enzima conversora de angiotensina tipo 2 (ECA2) por meio da ação da desintegrina e metaloprotease 17 (ADAM17) na hipertensão. Neste contexto, o ácido α-lipóico (AL), um potente antioxidante, vem sendo estudado por suas ações sobre o sistema cardiovascular e foi selecionado para o estudo na modulação da via Ang II/ROS/ADAM17/ECA2. Neste trabalho foram utilizados dois modelos de hipertensão dependentes de Angiotensina II (Ang II): dois-rins-um-clipe (2R1C) e o modelo desoxicorticosterona-sal (DOCA-sal). Primeiramente, ratos *Wistar* foram submetidos à cirurgia 2R1C ou Sham, para avaliação do tratamento crônico do AL sobre os parâmetros cardiovasculares. Quatro semanas após a indução da hipertensão renovascular, os ratos foram tratados com AL (60 mg/kg) ou solução salina durante 14 dias, por via oral. No 15º dia, a pressão arterial média (PAM) e freqüência cardíaca (FC) foram registradas. Além disso, o teste da sensibilidade do barorreflexo, utilizando fenilefrina (8 µg/kg, i.v) e nitroprussiato de sódio (25 µg/kg, i.v) foi realizado. O tratamento crônico com AL reduziu a pressão arterial em animais hipertensos, quando comparado ao grupo 2R1C + salina (133,5 ± 9,3 *vs* 176,5 ± 9,6 mmHg, $p<0,05$, respectivamente); no entanto, não foram observadas alterações significativas na FC. O tratamento com AL aumentou a sensibilidade de ambos os componentes simpático e parassimpático do barorreflexo (-2,80 ± 0,6 vs -2,61 ± 0,4 e -4,14 ± 0,6 vs -3,85 ± 0,5 bpm.mm Hg-1, $p<0,05$, respectivamente). Para investigar a relação entre ADAM17 e o estresse oxidativo, células Neuro2A foram tratadas com Ang II (100 nM) 24h após veículo ou AL (500 µM). Ang II aumentou a expressão da ADAM17 (100,2 ± 0,8 *vs* 120,5 ± 9,1%, $p<0,05$) e foi reduzida pelo tratamento com o AL (120,5 ± 9,1 *vs* 69,0 ± 0,3%, $p<0,05$). Em outro experimento, AL reduziu a expressão da ADAM17 (92,9 ± 5,3 *vs* controle: 77,3 ± 3,3%, $p<0,05$) em células que superexpressaram o ADAM17, bem como sua atividade foi atenuada após o tratamento com AL (109,5 ± 19,8 *vs* 158,0 ± 20,0 FU/min/mg de proteína, $p<0,05$). A produção de espécies reativas de oxigênio (ROS) mostrou-se aumentada em células com superexpressão da ADAM17 (114,1 ± 2,5 *vs* 101,0 ± 1,0%, $p<0,05$), porém em células tratadas com o AL, a formação de ROS foi atenuada (76,0 ± 9,1 *vs* 114,1 ± 2,5%, $p<0,05$). Em camundongos DOCA-sal, modelo em que a expressão e a atividade de ADAM17 encontram-se elevadas, a hipertensão foi diminuída pelo tratamento com AL (119,0 ± 2,4 *vs* 131,4 ± 2,2 mmHg, $p<0,05$). Além disso, o AL melhorou a disfunção autonômica e a sensibilidade do barorreflexo. O AL aboliu o aumento da expressão da NADPH-oxidase, bem como o aumento da atividade da ADAM17 e promoveu uma redução na atividade da ECA2 no hipotálamo dos animais DOCA-sal. Esses dados sugerem que o tratamento crônico com AL promove um efeito anti-hipertensivo, com melhora da sensibilidade do barorreflexo e função autonômica em ratos com hipertensão renovascular, bem como no modelo DOCA-sal. Portanto, podemos sugerir que o AL preserva a atividade compensatória da ECA2 por romper o ciclo de retroalimentação positiva entre a ADAM17 e o estress oxidativo, resultando na redução da hipertensão arterial.

**Palavras-chave**: ácido α-lipoico, Ang II, ADAM17, ECA2, barorreflexo, estresse oxidativo

# Abstract

**The hypotensive effect induced by α-lipoic acid in models of angiotensin II-dependent hypertension involves the inhibition of ADAM17.** QUEIROZ, T. M.; Tese de doutorado, Programa de pós-graduação em Produtos Naturais e Sintéticos Bioativos, PgPNSB/CCS/UFPB (2014)

## ABSTRACT

Oxidative stress has been indicated as a central mechanism in Ang II-dependent hypertension. Furthermore, previous studies reported that oxidative stress is associated with the impairment of angiotensin converting enzyme 2 (ACE2) compensatory activity by the disintegrin and metalloprotease 17 (ADAM17) during hypertension. In this context, α-lipoic acid (LA), a potent antioxidant, has been studied by its effects on cardiovascular system, however its effects on Ang II/ROS/ADAM17/ACE2 pathway have not been studied yet. Here we used two Ang II-dependent hypertensive models, the two-kidney-one-clip (2K1C) and deoxycorticosterone-sal (DOCA-salt) hypertension. Firstly, we analyzed the effects induced by chronic treatment with LA on blood pressure, heart rate and baroreflex sensitivity (sympathetic and parasympathetic components) in renovascular hypertensive rats. Male *Wistar* rats underwent 2K1C or sham surgery and were maintained untouched for four weeks to develop hypertension. Four weeks post-surgery, rats were orally treated with LA (60 mg/kg) or saline for 14 days. On the 15th day, mean arterial pressure (MAP) and heart rate (HR) were recorded. In addition, baroreflex sensitivity test using phenylephrine (8 μg/kg, i.v.) and sodium nitroprusside (25 μg/kg, i.v.) was performed. Chronic treatment with LA decreased blood pressure in hypertensive animals compared to 2K1C + saline group (133.5 ± 9.3 *vs* 176.5 ± 9.6 mmHg, $p<0.05$, respectively); however, no significant changes in baseline HR were observed. Regarding baroreflex, LA treatment increased the sensitivity of both the sympathetic and parasympathetic components (−2.80 ± 0.6 *vs* −2.61 ± 0.4 and −4.14 ± 0.6 vs. −3.85 ± 0.5 bpm.mm Hg−1, $p < 0.05$, n = 8, respectively). To investigate the relationship between ADAM17 and oxidative stress, Neuro2A cells were treated with Ang-II (100 nM) 24 h after vehicle or LA (500 μM). ADAM17 expression was increased by Ang-II (100.2 ± 0.8 *vs* 120.5 ± 9.1%, p<0.05) and decreased after LA (120.5 ± 9.1 *vs* 69.0 ± 0.3%, p<0.05). In other set of experiments, LA reduced ADAM17 (92.9 ± 5.3 *vs* control: 77.3 ± 3.3%, 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 the expression of NADPH oxidase subunits as well as the increase in ADAM17 and decrease in ACE2 activities in the hypothalamus of DOCA-salt hypertensive mice. Our data suggest that chronic treatment with LA promotes antihypertensive effect and improves baroreflex sensitivity and autonomic function in rats with renovascular hypertension as well as in DOCA-salt mice. Therefore, these data suggest that LA might preserve ACE2 compensatory activity by breaking the feed-forward cycle between ADAM17 and oxidative stress, resulting in reduction in hypertension.

**Key words**: α-lipoic acid, Ang II, ADAM17, ACE2, baroreflex, oxidative stress,

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**SUMÁRIO**

# Introdução

## 1 INTRODUÇÃO

O controle da pressão arterial (PA) é uma das funções fisiológicas mais complexas do sistema biológico, e dependente de ações integradas dos sistemas cardiovascular, renal, neural e endócrino (Campagnole-Santos; Haibara, 2001). Esses sistemas atuam por diferentes mecanismos, os quais promovem um maior afluxo sanguíneo para os tecidos em atividade e realizam uma redistribuição do sangue frente a situações de alteração na homeostase corporal (Lanfranchi; Somers, 2002). Alterações nesses sistemas de regulação podem induzir a distúrbios fisiológicos como a hipertensão arterial (Cain; Khalil, 2002).

A hipertensão arterial (HA) tem sido indicada como um fator de risco importante para a morbidade e mortalidade precoces causadas por doenças cardiovasculares (Simão et al., 2008). A HA representa um estado alterado da saúde no qual a pressão sanguínea está acima dos valores de 140/90 mmHg (Sociedade Brasileira de Hipertensão, 2010), resultado do desequilíbrio entre os vários mecanismos que contribuem para a sua regulação (Cain; Khalil, 2002).

A HA juntamente com as doenças cardiovasculares relacionadas são as principais causas de morte em países desenvolvidos e em desenvolvimento, causando um grande impacto na saúde humana. Neste cenário, a HA apresenta elevados custos médicos e socioeconômicos decorrentes principalmente das complicações que a acompanha, tais como: acidente vascular cerebral (AVC), doença arterial coronariana, insuficiência cardíaca e insuficiência renal crônica (Sociedade Brasileira de Hipertensão, 2010).

Diversos estudos relacionam a participação do aumento da atividade do sistema nervoso simpático no desenvolvimento da HA (Floras, 1992; Irigoyen et al., 2005; Wyss, 1993). Adicionalmente, relata-se que o aumento da atividade simpática e a redução da atividade parassimpática estão diretamente relacionados à disfunção do barorreflexo, uma vez que os barorreceptores modulam a atividade autonômica (Dall'ago et al., 1999; Botelho-Ono et al., 2011).

O papel dos barorreceptores na patogênese da HA tem sido sugerido desde os trabalhos preliminares de Hering (1927) e Heymans e Bouckaert (1930), que descreveram um aumento súbito da pressão arterial após a desnervação de áreas sinoaórticas (Campagnole-Santos; Haibara, 2001). Barorreceptores situados na camada adventícia do arco aórtico e seio carotídeo sentem variações na pressão arterial, propagando potenciais de ação para o Sistema Nervoso Central (SNC), que por sua vez desencadeiam ajustes autonômicos reflexos que tamponam as alterações da pressão arterial. Em condições patológicas, como na hipertensão, há prejuízo na regulação autonômica da pressão arterial, resultando em danos na sensibilidade do barorreflexo (GRASSI et al., 1998; SALGADO et al., 2009).

Dentre as regiões do SNC envolvidas no controle reflexo da pressão arterial, destacam-se o núcleo paraventricular do hipotálamo (PVN) e o bulbo ventrolateral rostral (RVLM). O primeiro é uma das principais áreas do SNC envolvidas no controle autonômico e neuro-humoral da pressão arterial (Burmeister et al., 2011), já que possui tanto neurônios simpáticos quanto neurônios vasopressinérgicos (Colombari et al., 2010; Xia et al., 2013). Os estímulos das vias da vasopressina provenientes do PVN contribuem para um

aumento da atividade simpática, que por sua vez induz uma elevação da pressão arterial (Antunes et al., 2006; Colombari et al., 2010). Além disso, a ativação de mecanismos que modulam a formação de Angiotensina II (Ang II) no PVN podem promover alterações na atividade e/ou reflexo simpático, ou ainda na sensibilidade do barorreflexo (Coote, 2007; Burmeister et al., 2011), tornando-se, portanto um órgão de grande interesse na investigação dos mecanismos de alteração da pressão arterial.

O RVLM é considerado uma área crítica para a geração da atividade vasomotora simpática e forma um componente central necessário para os reflexos cardiovasculares (Guo et al., 2009). A inibição de neurônios pré-motores simpáticos no RVLM por neurônios GABAérgicos do bulbo ventrolateral caudal (CVLM) constitui o fator inibitório necessário para o controle da eferência vasomotora simpática mediada pelo barorreflexo. Logo, a inibição desta via, bem como a remoção da inibição de neurônios do RVLM produz hipertensão aguda grave, consistente com o bloqueio dos reflexos barorreceptores (Sved et al., 2000).

O estresse oxidativo modula a função barorreflexa em vários processos fisiopatológicos (Monahan; Eskurza; Seals, 2004; Nishi et al., 2010). Por exemplo, a administração de sequestradores de radicais livres exógenos, tais como a superóxido dismutase e catalase, para o seio carotídeo de coelhos com aterosclerose induzida experimentalmente, aumenta a função barorreflexa (Li et al., 1996). Estes resultados sugerem um papel modulatório de espécies reativas de oxigênio sobre os barorreceptores carotídeos. Além disso, a infusão intravenosa aguda de ácido ascórbico, um conhecido antioxidante, aumenta a

sensibilidade do barorreflexo em pacientes com insuficiência cardíaca (Nightingale et al., 2003; Chapleau et al., 1995; Queiroz et al., 2012).

O estresse oxidativo tem sido apontado como um mecanismo fundamental na hipertensão arterial dependente de Ang II (Kitiyakara; Wilcox, 1998; Houston, 2005). ROS são importantes no aumento da pressão arterial, após administração de Ang II, a nível sistêmico ou diretamente no SNC. Considerando que a Ang II é composta por oito aminoácidos, tornando-a incapaz de atravessar a barreira hematoencefálica (BHE), os mecanismos que envolvem as ações da Ang II no cérebro que modulam a atividade simpática e induzem hipertensão arterial permanecem desconhecidos (Braga et al, 2008; Carvalho et al., 2012).

A hipótese mais aceita é a de que Ang II atua sobre neurônios em regiões específicas do cérebro, conhecidas como órgãos circunventriculares (CVOs), como por exemplo o órgão subfornical (SFO), área postrema (AP) e órgão vasculoso da lâmina terminal (OVLT), que são parcialmente ausentes de barreira hematoencefálica. Como resultado, a ativação dos CVOs induz uma produção local de Ang II em áreas cerebrais protegidas pela BHE, principalmente o RVLM, que por sua vez altera o tônus simpático levando a hipertensão (Carvalho et al., 2012). Outro importante fator é o de que componentes do sistema renina-angiotensina (SRA) estão presentes em várias regiões do cérebro que são envolvidas na regulação central da pressão arterial, incluindo o PVN, RVLM e o núcleo do trato solitário (NTS) (Davisson, 2003; Paton et al., 2001, 2007, 2008). Portanto, nessas regiões há formação local de Ang II e consequente aumento da pressão arterial. Entretanto, evidências mostraram que o processo inflamatório em vasos cerebrais, induzido por Ang

II, possui um papel fundamental no estágio inicial da hipertensão arterial. Ang II circulante induz a ativação de leucócitos, e ambos, os leucócitos intracapilares, como os leucócitos que transmigram a parede vascular, induzem a produção de citocinas pró-inflamatórias como a IL-1β, IL-6 e TNF-α, que são conhecidas por modularem a função neuronal, levando a simpatoexcitação (Shi et al., 2010; Waki et al, 2011).

O aumento nos níveis de Ang II em nível sistêmico quanto no SNC podem desencadear um aumento da atividade simpática e aumento da pressão arterial. Este fato foi comprovado por estudos que revelaram a hipertensão arterial induzida por Ang II, como um resultado causado por ambos os efeitos centrais quanto por elevações da PA periféricas. Estes efeitos promovem a ativação de células T e desenvolvimento de inflamação vascular. Esses achados suportam um mecanismo de retroalimentação positiva, no qual pequenas elevações da pressão arterial induzem ativação de células T, que por sua vez, desencadeiam inflamação e aumento da pressão arterial, levando a uma hipertensão arterial severa (Marvar et al., 2010). De acordo com um recente estudo desenvolvido por Biancardi et al. (2014), pôde-se notar que a Ang II, via ativação de receptores para Ang II tipo 1 (AT1R), induz um efeito inflamatório deletério sobre a BHE, promovendo o rompimento dessa barreira física, e assim permitindo o acesso desse hormônio em áreas do SNC normalmente protegidas, como o PVN, RVLM e o NTS.

A produção de Ang II é decorrente da ativação do SRA. Uma vez ativo, este sistema consiste em uma cascata enzimática formada de duas etapas, catalisadas pela renina e pela enzima conversora de angiotensina (ECA) (Min et al., 2009; Abadir, 2011). A renina atuará na conversão do angiotensinogênio

em um decapeptídeo, a angiotensina I. Este polipeptídeo apresenta características bastante instáveis, sendo rapidamente convertido por ação da ECA em Ang II.

Uma proteína recentemente identificada, e considerada um regulador negativo do SRA, a ECA2, cliva Ang I e Ang II em Ang 1-9 e Ang 1-7, respectivamente (Chang et al., 2011). Estudos mostram que a ECA2 está envolvida em mediar efeitos como a natriurese, vasodilatação e inibição da insuficiência cardíaca. Estes efeitos parecem estar relacionados com a redução do estresse oxidativo, uma vez que a deficiência de ECA2 induz um aumento de Ang II e ROS, mediado pela ativação do sistema NAD(P)H oxidase, com exacerbação do estresse oxidativo, levando a hipertensão (Chang et al., 2011; Bodiga et al., 2011; Xia et al., 2011; Xiao et al., 2011).

A primeira demonstração de uma ação biológica para a Ang 1-7 foi realizada por Schiavone et al. (1988) (Santos, Campagnole-Santos, Andrade, 2000). Ang 1-7 exibe um efeito antiarrítmico (Ferreira, Santos, Almeida, 2002), protege o coração de lesões induzidas por isquemia/reperfusão (Ferreira, Santos, Almeida, 2001), e preserva a função do ventrículo esquerdo após infarto do miocárdio induzida por isoproterenol (Loot et al., 2002). Além disso, Grobe et al. (2007) mostraram que 28 dias de infusão intravenosa de Ang 1-7 atenua a fibrose cardíaca induzida por acetato de desoxicorticosterona (DOCA-sal) ou hipertensão induzida por Ang II, sem alterar o grau de hipertrofia cardíaca ou a pressão arterial basal. A maior parte das ações induzidas por Ang 1-7 é mediada por seu receptor seletivo, o receptor Mas, acoplado a proteína G (Santos et al., 2003; Santiago et al., 2010). Adicionalmente, Ang 1-7

foi capaz de aumentar a sensibilidade do barorreflexo (Chaves et al., 2000; Santos, 2014).

A ECA2, que foi descoberta no ano 2000, é um exemplo de proteína integral de membrana, com um extenso domínio catalítico, uma simples hélice transmembrana e um domínio carboxiterminal curto. A ECA2 pode sofrer "*shedding"* por liberar o ectodomínio cataliticamente ativo da superfície celular para o meio extracelular (Lambert et al., 2005; Lai et al., 2011).

O termo *"Ectodomain shedding"* significa a liberação do domínio extracelular de uma proteína integral de membrana por proteólise (Arribas; Borroto, 2002). Este processo e as proteases envolvidas, denominadas *sheddases,* controlam a atividade biológica de proteínas membranares (Chow; Fernandez-Patron, 2007).

Um dos principais grupos das proteases *sheddases* é a família ADAM (*A Desintegrin And Metalloproteinase*). ADAM17 foi a primeira protease identificada desta família. Também conhecida como enzima conversora do fator de necrose tumoral α - TNFα (TACE), é constituída por um domínio amino terminal, um domínio catalítico, um domínio rico em cisteína (composto de desintegrina e domínios semelhantes ao fator de crescimento epidermal-EGF), além de um domínio transmembranar e citoplasmático.

ADAM17 tem uma distribuição ampla e funciona como *sheddase* para a clivagem proteolítica de várias proteínas da superfície das células, controlam a adesão celular, a transdução de sinal, a liberação de citocinas e fatores de crescimento (Garton et al., 2006; Buxbaum et al., 1998; Hao et al., 2004; Chow; Fernandez-Patron, 2007; Iwata; Enciso; Greenberg, 2009). Nesse contexto, a ADAM17 foi considerada, primariamente, a responsável pela liberação da

forma solúvel da ECA2. Estudos recentes demonstraram um aumento dos níveis circulantes de ECA2 em pacientes com insuficiência cardíaca, um achado que pode estar relacionado com o aumento do *shedding* desta proteína (Epelman et al., 2008; Iwata; Enciso; Greenberg, 2009).

A diversidade de proteínas que sofrem liberação do domínio extracelular indica que o processo é essencial não só durante o desenvolvimento, mas também em condições patológicas, incluindo câncer, artrite reumatoide, doença de Alzheimer e doenças inflamatórias (Garton et al., 2006; Li et al., 2006; Buxbaum et al., 1998; Hao et al., 2004).

Condições patológicas provocadas por Ang II, incluindo os fatores de risco cardiovasculares, são mediadas por ativação de metaloproteinases da família ADAM (Suzuki et al., 2005; Ohtsu et al., 2006). Ohtsu et al (2006) documentaram que a ADAM17 parece mediar a transativação do receptor do fator de crescimento epidermal (EGFR) e hipertrofia de células musculares lisas vasculares induzida por Ang II. Além disso, Xia et al (2013) observaram que a ligação de Ang II em receptores AT1, no cérebro, promove, não somente o aumento da pressão arterial, bem como uma suprarregulação da ADAM17, que por sua vez, irá proporcionar o *shedding* da ECA2. Dessa maneira, a ECA2 deixará de mediar a conversão de Ang II em Ang 1-7, que se liga à receptores Mas, com consequente diminuição da pressão arterial, reduzindo os níveis membranares desta enzima e aumentando sua forma solúvel, com perda de sua atividade compensatória sobre a pressão arterial (Figura 1). Nesse estudo foi possível identificar, pela primeira vez, que a inibição da expressão de ADAM17 previne a redução dos níveis de ECA2 no cérebro, e esse efeito está associado com a redução da hipertensão arterial.

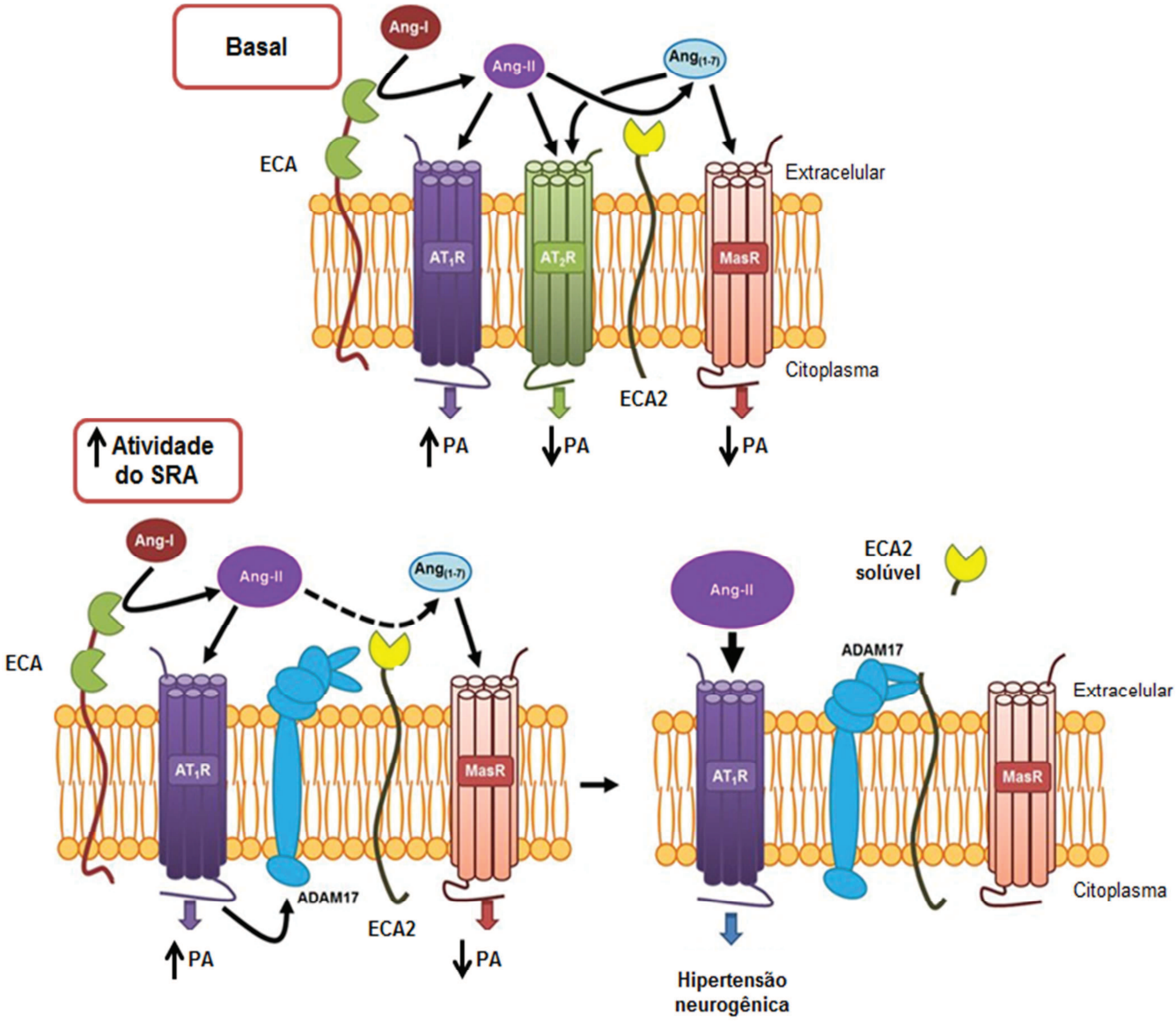


**Figura 1**. **Sistema renina angiotensina, ADAM17 e hipertensão neurogênica**. Em condições fisiológicas (basais), a Ang II, formada a partir de Ang I, pode ligar-se a receptores AT1 e AT2 induzindo um aumento e diminuição da PA, respectivamente. Além disso, Ang 1-7 atua em receptores Mas induzindo uma diminuição da PA. Entretanto em casos em que haja uma aumento da atividade do SRA, a Ang II se liga ao seu receptor AT1 e promove a ativação da ADAM17. Uma vez a ADAM17 no seu estado ativo, irá funcionar como uma *sheddase* e promover a clivagem de proteínas membranares, como a ECA2. Esta enzima em seu estado solúvel não irá favorecer a formação de Ang 1-7 e seu efeito compensatório será interrompido, favorecendo a hipertensão. (Ang I: angiotensina I; Ang II: angiotensina II; AT1R: receptor para angiotensina tipo 2; ECA2: enzima conversora de angiotensina tipo 2; MasR: receptor específico para Ang 1-7, receptor Mas; ADAM17: desintegrina e mataloprotease 17).
(Modificado de Xia et al., 2013)

As espécies reativas de oxigênio também são capazes de ativar a proteína ADAM17, por exemplo, Brill e colaboradores (2009) mostraram, *in vitro*, que o estresse oxidativo ativa ADAM17 em plaquetas, e essa ativação representa um mecanismo limitante da função plaquetária.

Na tentativa de reduzir o estresse oxidativo e propiciar os efeitos benéficos causados por essa redução, a terapia antioxidante faz-se necessária e vem sendo utilizada para o tratamento de várias enfermidades, incluindo a hipertensão arterial (Chen et al., 2001; Costa et al., 2009).

Um antioxidante que vem sendo estudado nos últimos anos é o ácido α-lipóico (AL), também conhecido como ácido 1,2-ditiolano-3-pentanóico, o qual é um antioxidante dissulfeto com um centro quiral que existe em ambas as formas enantioméricas R- e S-. No entanto, apenas a forma R-AL (Figura 2) é conjugada com resíduos conservados de lisina em uma ligação amida, tornando assim esta isoforma essencial como um cofator em sistemas biológicos (Reed, 1974; Shay et al., 2009). O ácido α-lipóico funciona como um cofator nos complexos multienzimáticos que catalisam a descarboxilação oxidativa do α-cetoglutarato e piruvato desidrogenases, enzimas essenciais na produção final de ATP (Reed, 1974; Takaoka et al., 2001).

O ácido α-lipóico e sua forma reduzida, ácido diidrolipóico, reduzem o estresse oxidativo pelo sequestro de um número de radicais livres nos domínios membranares e aquosos (Maritim et al., 2003). Além disso, este antioxidante tiol (formas oxidada e reduzida) tem uma capacidade quelante de metais, como por exemplo, os íons $Cu^{2+}$, $Zn^{2+}$, $Pb^{2+}$. AL é capaz de regenerar antioxidantes endógenos, tais como as vitaminas C e E e também aumenta a atividade do óxido nítrico (NO). O ácido α-lipóico mostrou-se ainda ser eficaz

em melhorar a plasticidade e regeneração neuronal (Wang et al., 2011), aumentar a função endotelial em pacientes com diabetes tipo II e reduzir o tamanho do infarto no modelo de injúria e reperfusão (Biewenga et al., 1997; Heinisch et al., 2010; Connell et al., 2011).

Os resultados obtidos com o estudo do ácido lipóico têm sido bastante promissores, possibilitando a pesquisa desse antioxidante na suplementação alimentar e no tratamento da hipertensão. Assim, baseado nas importantes propriedades do ácido α-lipóico, optou-se por estudar este antioxidante, pois os mecanismos de ação envolvidos nas respostas cardiovasculares promovidas pelo ácido lipóico ainda não foram totalmente esclarecidos. Nesse contexto, o presente estudo tem como hipótese investigar a relação entre Ang II, ECA2, ADAM17 e ROS, utilizando o antioxidante ácido α-lipóico.

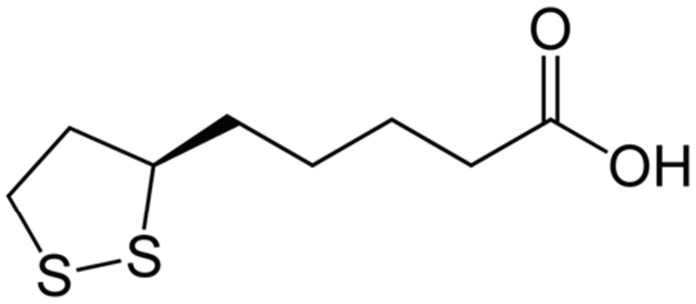


**Figura 2**. Estrutura química do ácido *R* α-lipóico

# 2 OBJETIVOS

## *2.1 Objetivo Geral*

Investigar o mecanismo anti-hipertensivo do ácido α-lipóico (AL) na modulação da ADAM17 por espécies reativas de oxigênio (ROS), em dois modelos de hipertensão arterial dependentes de angiotensina II (dois-rins-um-clipe (2R1C) e DOCA-sal).

## *2.2 Objetivos específicos*

- Caracterizar os possíveis efeitos anti-hipertensivos do AL, frente ao modelo experimental de hipertensão renovascular (2R1C), sobre a pressão arterial e a frequência cardíaca em ratos não-anestesiados, bem como o efeito do antioxidante sobre a sensibilidade do barorreflexo;
- Estudar a influência do estresse oxidativo na modulação da ADAM17 em modelos *in vivo* (analisando a sensibilidade do barorreflexo e a atividade autonômica) utilizando camundongos C57BL/J6 hipertensos DOCA-sal;
- Avaliar a atividade das proteínas ECA2 e ADAM17 no hipotálamo de animais normotensos e hipertensos tratados com o ácido lipóico, assim como investigar a participação de enzimas envolvidas na produção de ROS (NADPH oxidase);
- Analisar a expressão e atividade da ADAM17, bem como quantificar o estresse oxidativo (ROS) em culturas de células Neuro2A, utilizando técnicas bioquímicas na presença e/ou ausência de AL.

# Resultados e Discussão

**3.1 Artigo 1:** *α-Lipoic Acid Reduces Hypertension and Increases Baroreflex Sensitivity in Renovascular Hypertensive Rats. Thyago M. Queiroz, Drielle D. Guimarães, Leônidas G. Mendes-Junior and Valdir A. Braga, Molecules 2012, 17, 13357-13367.*

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*Article*

# α-Lipoic Acid Reduces Hypertension and Increases Baroreflex Sensitivity in Renovascular Hypertensive Rats

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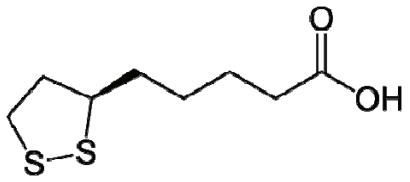
**Abstract:** Renovascular hypertension has robust effects on control of blood pressure, including an impairment in baroreflex mechanisms, which involves oxidative stress. Although α-lipoic acid (LA) has been described as a potent antioxidant, its effect on renovascular hypertension and baroreflex sensitivity (BRS) has not been investigated. In the present study we analyzed the effects caused by chronic treatment with LA on blood pressure, heart rate and baroreflex sensitivity (sympathetic and parasympathetic components) in renovascular hypertensive rats. Male Wistar rats underwent 2-Kidney-1-Clip (2K1C) or sham surgery and were maintained untouched for four weeks to develop hypertension. Four weeks post-surgery, rats were treated with LA (60 mg/kg) or saline for 14 days orally. On the 15th day mean arterial pressure (MAP) and heart rate (HR) were recorded. In addition, baroreflex sensitivity test using phenylephrine (8 μg/kg, i.v.) and sodium nitroprusside (25 μg/kg, i.v.) was performed. Chronic treatment with LA decreased blood pressure in hypertensive animals; however, no significant changes in baseline HR were observed. Regarding baroreflex, LA treatment increased the sensitivity of both the sympathetic and parasympathetic components. All parameters studied were not affected by treatment with LA in normotensive animals. Our data suggest that chronic treatment with LA promotes antihypertensive effect and improves baroreflex sensitivity in rats with renovascular hypertension.

**Keywords:** 2K1C; blood pressure; reactive oxygen species; lipoic acid; parasympathetic; sympathetic

## 1. Introduction

α-Lipoic acid (LA), also known as 1,2-dithiolane-3-pentanoic acid or thioctic acid, is a disulfide antioxidant with one chiral center that 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 (Figure 1) [1,2]. Among its properties, α-lipoic acid functions as a cofactor in the multienzyme complexes that catalyze the oxidative decarboxylation of α-ketoglutarate and pyruvate dehydrogenase, critical enzymes in ATP production [1,3]. LA is also absorbed intact from dietary sources, and it can be accumulated in tissues. There is evidence that orally supplied LA might not be used as a metabolic cofactor but instead, elicits important activities with potential therapeutic value [2].

**Figure 1.** Chemical structure of (*R*)-α-lipoic acid.




LA and its reduced form, dihydrolipoic acid, reduce oxidative stress by scavenging a number of free radicals in both membrane and aqueous domains [4]. In addition, this thiol antioxidant, which holds a metal chelating capability, is able to regenerate endogenous antioxidants such as vitamins C and E and also increases nitric oxide (NO) activity [5–7].

Increased oxidative stress and associated oxidative damage are considered mediators of vascular injury in cardiovascular pathologies, including hypertension and atherosclerosis [8,9]. The relationship between oxidative stress and hypertension has been demonstrated in several models of experimental hypertension. For example, increased reactive oxygen species (ROS), including superoxide ($O_2^{\bullet -}$), hydroxyl ($OH^{\bullet}$) and peroxyl radicals ($ROO^{\bullet}$) or hydrogen peroxide ($H_2O_2$), formation precedes the development of hypertension in spontaneously hypertensive rats (SHR) and in angiotensin II (Ang II)-infused mice [10–13]. In this latter model, the ROS production is dependent on NADH/NADPH, a plasma membrane-bound protein that, when activated by binding of Ang II in $AT_1$ receptors, produces ROS [14,15].

Hypertension and cardiovascular diseases are the leading cause of death in developed and developing countries [16], causing a great impact on global human health. In an attempt to reduce this impact, in recent decades several research groups have worked extensively to improve the treatment of cardiovascular diseases including the discovery of new therapy strategies and drugs [17,18]. Among these, we can highlight the use of an antioxidant therapy such as ROS scavengers and vitamins,

superoxide dismutase (SOD) mimetics or NADPH oxidase inhibitors such as apocynin, to attenuate or prevents the development of hypertension [19–22].

It is well established that alterations in blood pressure is direct associated with changes in the baroreceptor reflex function. Baroreceptors situated in the aortic arch and carotid sinuses sense variations in blood pressure and trigger reflex autonomic adjustments that buffer alterations in blood pressure. In pathological conditions such as hypertension, there is impairment in the autonomic regulation of blood pressure resulting in damage in baroreflex sensitivity [23,24].

In view of the fact that reactive oxygen species seems to play an important role in autonomic control during health and disease and that effects of antioxidant therapy involved in baroreflex control are not completely understood, in the present study we tested the hypothesis that chronic administration of α-lipoic acid, a key antioxidant, would promote an antihypertensive effect and improve baroreflex sensitivity in renovascular hypertensive rats.

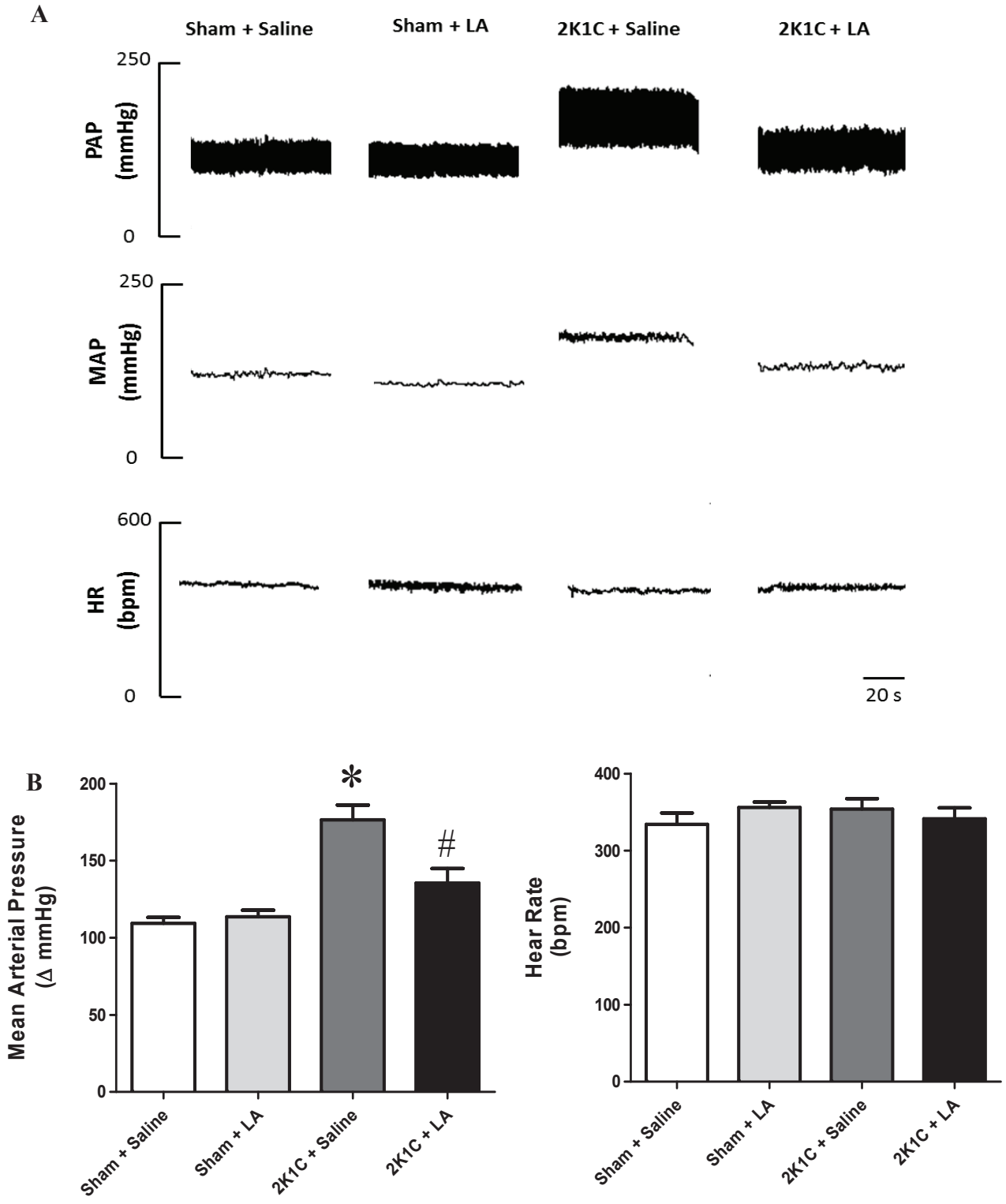
## 2. Results and Discussion

### *2.1. Renal Artery Clipping Induces Hypertension without Affecting Heart Rate*

Four weeks after clipping of the kidney, rats from the 2K1C + Saline group (n = 8) were hypertensive when compared to the sham-operated group (n = 8), (176.5 ± 9.6 *vs*. 109.3 ± 3.8 mmHg, respectively, $p < 0.05$). However in the 2K1C + LA group (n = 7) we did not observe differences in relation to the Sham + LA group (n = 6), (133.5 ± 9.3 *vs*. 113.6 ± 4.2 mmHg, respectively). In addition rats from the 2K1C + LA group presented a reduction in blood pressure when compared to 2K1C + saline group (133.5 ± 9.3 *vs*. 176.5 ± 9.6 mmHg, respectively), as shown in the representative tracings and in the group data in Figure 2A,B.

These results corroborate other studies which have been reported that common antioxidants for clinical use and experimental trials such as ascorbic acid (AAC) and *N*-acetylcysteine (NAC) were able to delay or prevent the development of different types of hypertension. When compared the intensity of the reduction in blood pressure, we observed that the hypotensive effect promoted by LA was similar to values found from the previous studies [13,25]. Indeed, known to scavenge hydroxyl radicals, singlet oxygen, hydrogen peroxide, hypochlorous acid, peroxynitrite and nitric oxide. The present investigation is the first description of antihypertensive effect of LA in the renovascular hypertension model. This finding has important clinical significance because the Goldblatt 2K1C experimental model of renovascular hypertension is characterized by excess of the activity of the rennin-angiotensin-aldosterone system (RAAS) and increased circulating levels of angiotensin-II, which is identified as the main mediator of the mechanisms involved in the essential hypertension [26]. These include vasoconstriction, induction of vascular smooth muscle cell growth [27,28], stimulation of proto-oncogene expression [29–31], modulation of myocardial hypertrophy and fibrosis [32–35] and modulation of ventricular remodeling after myocardial infarction [15]. Several evidences suggest that a key mechanism by which angiotensin-II influences blood pressure is via its ability to stimulate the production of reactive oxygen species [12,14], mainly superoxide anion [36,37], via activation of NADPH oxidase.

**Figure 2.** (**A**) Representative tracings showing the changes in pulse arterial pressure (PAP, mmHg), mean arterial pressure (MAP, mmHg), and heart rate (HR, bpm) of a rat from Sham + Saline; Sham + LA; 2K1C + Saline and 2K1C + LA groups four weeks after clipping the right renal artery. (**B**) MAP and HR values of Sham + Saline (n = 6, open bar); Sham + LA (n = 6, light grey bar); 2K1C + Saline (n = 6, dark grey bar) and 2K1C + LA (n = 6, black bar) groups. * $p < 0.05$, when compared to Sham + Saline group. # $p < 0.05$, when compared to 2K1C + Saline group.



NADPH oxidase is an enzyme composed of two membrane-bound subunits (gp91phox and p22phox), cytoplasmic subunits (p40phox, p47phox, and p67phox), and the small G protein Rac1a [38]. After activation of AT-1 receptors by angiotensin-II, the cytoplasmic subunits bind to the membrane subunits and activate the enzyme, resulting in the intracellular production of superoxide. The increase of superoxide leads to changes in ion channels, particularly calcium and potassium channels, altering neuronal firing properties in areas of the brain such as the rostral ventrolateral medulla (RVLM) resulting in increase in sympathetic nerve activity and increase in blood pressure [39]. Besides of blood pressure measurement, heart rate was also evaluated in this study. Interestingly, chronic administration of α-lipoic acid did alter baseline heart rate in normotensive or hypertensive rats as illustrated in Figure 2.

### *2.2. Chronic Administration of LA Restores Baroreflex Sensitivity in Renovascular Hypertension*

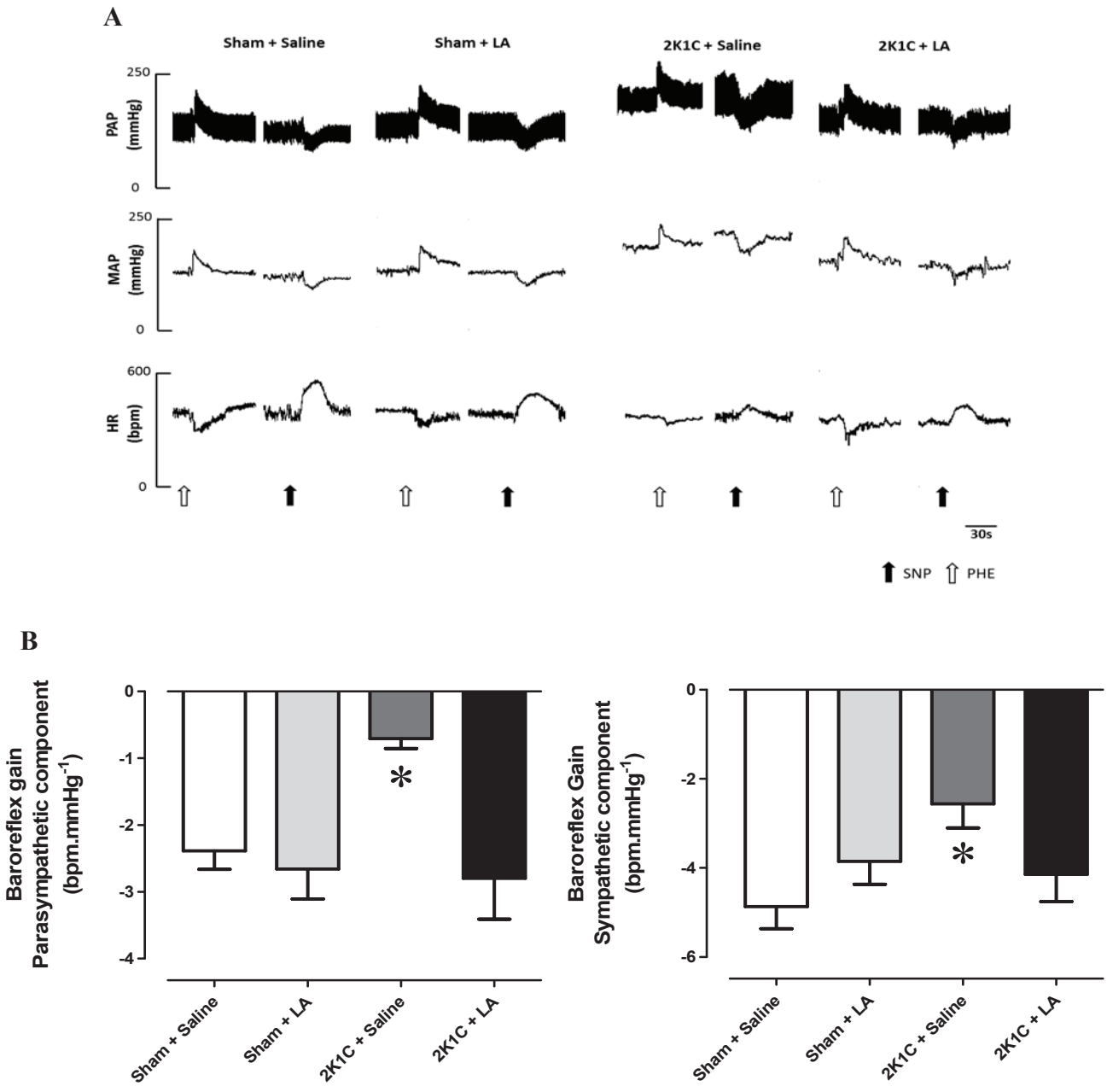
In order to investigate the baroreflex sensitivity, after baseline MAP and HR recordings, animals received intravenous injections of phenylephrine (Phe, 8 μg/kg) and sodium nitroprusside (SNP, 25 μg/kg) to determine the baroreceptor reflex responses. Acute administration of Phe was used to evaluate the parasympathetic component and SNP to evaluate the sympathetic autonomic component of the baroreflex.

Representative traces showing the changes in heart rate in response to baroreflex activation are illustrated in Figure 3A. Rats from the 2K1C + Saline group presented a reduction in baroreflex sensitivity in both parasympathetic and sympathetic components when compared to the Sham + Saline group (−0.70 ± 0.1 *vs.* −2.38 ± 0.2 and −2.56 ± 0.5 *vs.* −4.86 ± 0.5 bpm.mmHg$^{-1}$, respectively, $p < 0.05$, n = 8) as shown in Figure 3B. In 2K1C rats, chronic treatment of LA (60 mg/Kg, v.o.), restored the depressed baroreflex sensitivity to values found by sham-operated rats treated with LA in both parasympathetic and sympathetic component of baroreflex system (−2.80 ± 0.6 *vs.* −2.61 ± 0.4 and −4.14 ± 0.6 *vs.* −3.85 ± 0.5 bpm.mm Hg$^{-1}$, $p < 0.05$, n = 8).

There is convincing experimental evidence that oxidative stress modulates baroreflex function in health and disease [40,41]. For example, administration of exogenous free radical scavengers, such as superoxide dismutase and catalase, to the carotid sinus in rabbits with experimentally induced atherosclerosis improves the depressed baroreflex function [42]. These results suggest an inhibitory role of reactive oxygen species on carotid baroreceptors. In addition, acute intravenous infusion of ascorbic acid, a well-known antioxidant, increases baroreflex sensitivity in patients with cardiac dysfunction [43,44].

According to these related studies, as well as in the present investigation, administration of antioxidants had no effect on baroreflex function in normotensive animals [42,43], suggesting that anti-oxidant therapy in the absence of oxidative stress has no influence on baroreflex sensitivity. Mutually, the results from the present study and those from previous findings support the insight that oxidative stress reduces baroreflex sensitivity and that anti-oxidant therapy, such as α-lipoic acid, could restore it.

**Figure 3.** Baroreflex sensitivity test. (**A**) Representative tracings from each of the four groups (Sham + Saline; Sham + LA; 2K1C + Saline and 2K1C + LA) showing the changes in pulse arterial pressure (PAP, mmHg), mean arterial pressure (MAP, mmHg), and heart rate (HR, bpm) in response to phenylephrine (black arrows) and sodium nitroprusside (open arrows). (**B**) Values for baroreflex sensitivity (bpm.mmHg$^{-1}$) determined by the modified Oxford method using intravenous injection of NPS and Phe in Sham + Saline (n = 6, open bar); Sham + LA (n = 6, light grey bar); 2K1C + Saline (n = 6, dark grey bar) and 2K1C + LA (n = 6, black bar) groups. * $p < 0.05$, when compared to Sham + Saline group.




Although our data suggest that the antioxidant-mediated improvement in baroreflex sensitivity observed in 2K1C rats is probably due to increase in autonomic function, it is not possible to determine the exact mechanism at which LA exerts its positive influence on baroreflex function in renovascular hypertensive rats. Evidence from other experimental studies has suggested that diminished baroreflex

sensitivity is caused by endothelial dysfunction (ED) [44]. Because decreasing the synthesis and release of NO is the main factor to promote ED, studies has been shown that LA improves endothelial NO synthesis and thus improving endothelial function [45]. In particular, in experimentally induced endothelial dysfunction, the decreased prostacyclin and increased thromboxane concentrations are associated with reduced baroreflex impulses from the carotid artery [44]. Moreover, experimental evidence strongly suggests a direct suppressive influence of ROS on baroreceptors (*i.e.*, a peripheral site of action) [42]. The enzyme NADPH oxidase has been suggested as a possible target to increase ROS generation. A number of drugs used for the treatment of hypertension, hypercholesterolaemia and coronary artery disease such as the statins, AT1 (angiotensin II receptor type 1) antagonists and ACE inhibitors have been shown to decrease NADPH oxidase-derived superoxide and ROS production [46–48]. These therapies could repair the pro-oxidants levels and reestablish the regular conditions of the baroreflex sensitivity as well as cardiovascular parameters in the renovascular hypertension.

## 3. Experimental

### *3.1. Animals*

Twenty-seven adult male Wistar rats (280–330 g) were housed in conditions of controlled temperature (21 ± 1 °C) and exposed to a 12 h light-dark cycle with free access to food (Labina®, Purina, São Paulo, Brazil) and tap water. All procedures described in the present study are in agreement with Institutional Animal Care and Use Committee of the Federal University of Paraiba (CEPA/LTF protocol nº 0305/09).

### *3.2. Surgical Procedures*

Rats underwent surgical procedures in order to develop renovascular hypertension (2K1C model) as described by Botelho-Ono *et al.* [13]. Briefly, under combined ketamine and xylazine anesthesia (75 and 10 mg/kg, i.p., respectively), a midline abdominal incision was made. The right renal artery was exposed and isolated over a short segment by blunt dissection. A U-shaped silver clip (0.2 mm internal diameter) was placed over the vessel at a site proximal to the abdominal aorta and the wound closed and sutured. A sham procedure, which entailed the entire surgery with the exception of renal artery clipping, served as control. After the procedures, rats returned to their home cages and remained untouched for six weeks in order to develop hypertension.

### *3.3. Blood Pressure and Heart Rate Recordings*

After six weeks, rats were anesthetized with ketamine and xylazine (75 and 10 mg/kg, i.p., respectively), polyethylene catheters inserted into the lower abdominal aorta and inferior vena cava through left femoral artery and vein, respectively. Both catheters were filled with heparinized saline, tunneled subcutaneously, exteriorized and sutured at the dorsal surface of the neck. Twenty-four hours after surgical procedures, experiments were performed in conscious rats as previously described [49]. Blood pressure and heart rate were recorded using a pressure transducer (MLT0380/D, ADInstruments, Sydney, Australia) and connected to a computer (Mikro-tip blood pressure system, ADInstruments) running the LabChart software (ADInstruments).

### *3.4. Baroreflex Sensitivity Test*

For baroreflex sensitivity studies, animals were divided in four different groups: Sham + Saline and Sham + LA, 2K1C + Saline, 2K1C + LA. Four weeks after the clip implantation, animals were treated with a daily dose of Lipoic Acid (60 mg/kg, v.o.) or Saline for fourteen days. At the day of the experiment, following baseline blood pressure and heart rate recordings, baroreflex was activated using classical vasoactive drugs (modified Oxford Method, Braga *et al.*) [50] before and after bolus acute administration of vasoactive drugs: Phenylephrine (8 μg/kg) and sodium nitroprusside (25 μg/kg). A 15-minute interval as allowed between phenylephrine and sodium nitroprusside injections. Reflex changes in heart rate produced by vasoactive drugs administration were quantified and plotted as changes in heart rate over changes in mean arterial pressure (ΔHR/ΔMAP) as described by Braga *et al.* [50] All data from each group were analyzed by linear regression using the GraphPad Prism software and the slope of the linear regression yield baroreflex gain for each group.

### *3.5. Statistical Analyses*

Results are expressed as mean ± SEM. Data were analyzed by Student's t test or two-way repeated measures analysis of variance (ANOVA) followed by a Dunnett post-test for multiple comparisons whenever appropriate. All statistical analyses were performed using GraphPad Prism (v. 5.0, GraphPad Software, Inc.). Statistical significance was defined as $p < 0.05$.

## 4. Conclusions

In summary, our results suggest that antioxidant therapy by chronic treatment with α-lipoic acid reduces hypertension and improves baroreflex sensitivity in rats with renovascular hypertension. Despite the precise site of action where the chronic anti-oxidant treatment produces its beneficial effects remains unknown, it seems to involve both peripheral and central mechanisms, since LA has an amphipathic structure and thus can cross the blood-brain barrier.

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*Sample Availability*: Samples of the compounds are available from the authors.

**3.2 Artigo 2:** *α-Lipoic acid reduces neurogenic hypertension by blunting ADAM17-mediated increase in oxidative stress. Thyago M. de Queiroz, Huijing Xia, Valdir A. Braga and Eric Lazartigues. Artigo a ser submetido ao periódico Hypertension.*

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

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Short title: ADAM17 and Oxidative Stress in Hypertension

## 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 (100.2 ±0.8 *vs.* 120.5 ±9.1%, $p<0.05$) and decreased after LA (120.5 ±9.1 *vs.* 69.0 ±0.3%, $p<0.05$). In other set of experiments, LA reduced ADAM17 (92.9 ±5.3 *vs.* control: 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 the expression of NADPH oxidase subunits 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, Angiotensin II

## 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 angiotensin II receptor ($AT_1R$). Therefore, Ang-II acts as a potent vasoconstrictor, regulates water intake and salt metabolism and increases sympathetic outflow and BP (Werner et al., 2008; Xia et al., 2013).

Abundant evidence suggests that a key mechanism by which Ang-II contributes to hypertension is by increasing oxidative stress (Romero and Reckelhoff, 1999; Zimmerman et al., 2004). Oxidative stress is a condition of increased production of reactive oxygen species (ROS) and/or reduction of scavenging mechanisms (Xia et al., 2011). 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 (Zimmerman et al., 2004; Gao et al., 2004; Wang et al., 2004; Burmeister et al., 2010; Xia et al., 2011). 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 (Chang et al., 2011). 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 RAS promotes vasodilation, anti-proliferation and reduction of heart failure (Ferrario et al., 2005; Santos et al., 2003; Xu et al., 2011).

ACE2 is a membrane protein which can undergo shedding to release a catalytically active ectodomain from the cell surface into the extracellular milieu (Lambert et al., 2005; Lai et al., 2011). The proteases involved in this process are called sheddases and they control the biological activity of membrane proteins (Chow & Fernandez-Patron, 2007). A well-known sheddase is a disintegrin and metalloproteinase 17 (ADAM17), also called tumor necrosis factor-(TNF-α)-converting enzyme (Iwata et al., 2009).

Studies have showed that ADAM17 mediates transactivation of the epidermal growth factor receptor (EGFR) and hypertrophy of vascular smooth muscle cells induced by Ang-II (Ohtsu et al., 2006). Other *in vitro* studies demonstrated that ROS are able to activate ADAM17 in platelets and this effect could characterize a limiting mechanism for platelet function (Brill et al., 2009). Furthermore Zeng et al. (2013) have shown that Nox4-mediated ROS production is required to increase ADAM17 expression and subsequent induction of cardiac hypertrophy.

Therefore, we hypothesized that chronic RAS activation enhances oxidative stress and ADAM17 activity thus promoting ACE2 shedding. To test this hypothesis, we used a new 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 thioctic acid, is a disulfide antioxidant with one chiral center that 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 (Reed 1974; Shay et al, 2009). We recently documented that LA reduces BP and improves baroreflex sensitivity in renovascular hypertensive rats (Queiroz et al, 2012). 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) (Schenk; McNeil, 1992; Yemane et al., 2010).

Our data show that LA blunts ADAM17 overexpression by reducing oxidative stress in Neuro2A cells. Using DOCA-salt hypertensive mice, we demonstrated that LA prevents DOCA-salt-induced hypertension. LA also improves baroreflex sensitivity and autonomic function in this model. In addition, the increase in brain ADAM17 activity and resulting decrease in ACE2 activity were reduced by LA in DOCA-salt hypertensive mice. Finally, we observed that LA reduces the increased brain expression of Nox2 and Nox4 in DOCA-salt hypertensive mice.

## Material and Methods

An expanded Materials and Methods section is available in the online data supplement (please see http://hyper.ahajournals.org).

### Animals

Experiments were performed in adult (6 to 9 weeks of age; 25 to 30 g) C57BL/6J mice (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 described previously (Xia et al., 2013 ). Treatment groups included: Sham (n=6); DOCA (n=6); DOCA + TEMPOL (258 mg/kg/day; n=6) and DOCA + LA (600 mg/kg/day; n=6).

Cell culture and transfection

Neuro-2A cells (ATCC) were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 10 units/mL penicillin and 100 μg/mL streptomycin. The cells were transiently transfected using Lipofectamine 2000 reagent (Invitrogen), in Dulbecco's modified Eagle's medium with no antibiotics and fetal bovine serum at ~80% confluence according to manufacturer's instructions. After 6 h, the cells were trypsinized and plated at the desired density in full medium for the next 30 h. The cells were serum-starved 24 h before each experiment.

Statistics

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 (Xia et al., 2013). 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 (100.2 ±0.8 *vs.* 120.5 ±9.1%, p<0.05, Figure 1A). This increase was reduced by pretreatment with either LA (120.5 ±9.1 *vs.* 69.0 ±0.3%, *P*<0.05, Figure 1A) or tempol (118.4 ±7.0 *vs.* 87.0 ±0.7%, *P*<0.05, Figure 1B). These data confirm the Ang-II-mediated up-regulation of neuronal ADAM17, suggest a critical role for oxidative stress in this process and establish LA as an effective antioxidant.

*LA reduces ADAM17 overexpression and activity in Neuro2A cells*

In order to clarify the mechanisms involved in LA-mediated reduction of ADAM17 expression, the following experiments were performed in cells transfected with a plasmid overexpressing ADAM17. Western Blotting analysis revealed that transfection with the ADAM17 plasmid lead to ~50% increase in the sheddase's expression in Neuro2A cells compared to control (155.2 ± 17.8 *vs* 100.0 ±11.2 %, *P*<0.05, Figure 2A). ADAM17 overexpression was significantly blunted after 24 hours of LA treatment (92.9 ±5.3 *vs.*155.2 ±17.8 %, *P*<0.05, 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 ~2-3-fold increase in ADAM17 activity (158.0 ±20.0 *vs.* 77.3 ±3.3 FU/min/µg protein, p<0.05, Figure 2C). Interestingly, 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 leads to a gradually developing pressor response that is a model of neurogenic hypertension. DOCA-salt treatment elicited a rapid rise in MAP within 48 hours (119.4 ±2.7 mmHg) that reached a plateau in the 2$^{nd}$ 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) 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 and 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 reduction of sympathetic drive and enhancement of vagal tone) although LA exhibited a more modest effect on vascular sympathetic drive, with a tendency to reduce the vascular tone. To

determine the role of antioxidant therapy in DOCA-salt hypertension mice, we assessed spontaneous baroreflex sensistivity (SBRS) using the sequence method (Feng, 2010; Xia, 2010). 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.* 2.2 ±0.3 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 (Xia et al., 2013). Accordingly, ADAM17 activity was increased by 25% and ACE2 activity was reduced by 20% after 3 weeks in the hypothalamus of DOCA-salt hypertensive mice ($P<0.05$ *vs.* sham, Figure 4). Interestingly, ADAM17 activity was blunted by tempol (366.1 ±27.0 *vs.* 546.8 ±14.0 FU/min/µg protein, $P<0.05$ Figure 4A) or LA (445.0 ±0.9 *vs.* 546.8 ±14.0 FU/min/µg protein, $P<0.05$ Figure 4A). In addition, tempol (103.1 ±9.3 *vs.* 80.8 ±3.3 %FU/min/µg protein, $P<0.05$ Figure 4B) and LA (97.8 ±4.5 *vs.* 80.8 ±3.3 %FU/min/µg protein, $P<0.05$ Figure 4B) prevented the reduction in ACE2 activity Our data suggest that the reduction of oxidative stress by LA and tempol restore 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 2D, oxidative stress was increased in DOCA-salt hypertension, and this was associated with increased protein expression for Nox2 (367.1 ±71.0 *vs.* 100.0 ±21.0%, $p<0.05$, Figure 5A and B) and Nox4 (172.3 ±20.1 *vs.* 100.0 ±14.4%, $p<0.05$, Figure 5A and 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.

Here we report that Ang-II treatment in Neuro2A cells induces an increase in protein expression for ADAM17. Increase in ADAM17 expression has been showed in several models of cardiovascular diseases, including atherosclerosis (Saito et al., 2001), hypertension (Griendling et al., 1997) and aortic aneurysm (Eguchi et al., 1998). Many mechanisms have been proposed by which Ang-II induces ADAM17 activation. An important factor involved in ADAM17 stimulation is through ROS production (Brill et al., 2009; Patel et al., 2014). Strong evidence points out that ROS stimulates the removal of the inhibitory pro-domain of ADAMs (Zhang et al., 2001), and this fact leads to activation of ectodomain shedding (Hino et al., 1999; Sanderson et al., 2006). ROS also could mediates the phosphorylation of p38 MAP kinase (MAPK), which in turn phosphorylates and activates the threonine-735 residue in ADAM17 protein (Xu, Derynck, 2010; Lambert et al., 2008; Patel et al., 2014). However according Sanderson et al. (2006), the well-known antioxidant N-acetyl-L-cysteine impaired metalloprotease-dependent ectodomain shedding by prevent ROS-mediated ADAM17 activation. Our study is in agreement with previous findings since LA not only reduced ADAM17 overexpression as well as blunted ADAM17 activity, suggesting an inhibitory response in ADAM17 expression/activity due to an antioxidant effect.

Ang-II induces ROS production, including superoxide ($O_2^{\cdot-}$), in neurons of cardiovascular regulatory brain areas such as the paraventricular nucleus of the hypothalamus, the subfornical organ and the rostral ventrolateral medulla (Zimmerman et al., 2004a and b; Braga, 2010, Braga et al, 2011; Burmeister et al, 2011), and decrease ACE2 expression in Neuro2A cells (Xia et al., 2011). These alterations could be 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, corroborating with previous studies. On the other hand, previous data show that adenovirus-mediated ACE2 overexpression reduces ROS formation (Feng et al., 2008). Furthermore, ACE2 depletion could lead to a raise in ROS production, thereby Xia et al (2011) named ACE2 activation as a new “antioxidant” effect. From our knowledge, this 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 (Garwitz and

Jones, 1982), depending on high salt intake and reduced renal mass, leading to hypervolemia. Excess of mineralocorticoid leads to an imbalance in renal sodium handling by increasing sodium and water reabsorption (Guyton et al.1972; Yemane et al., 2010). Notably, after chronic DOCA-salt treatment the peripheral RAS, a major regulator of BP, is suppressed (Gavras et al., 1975), however, in the brain, there is an overactivity of RAS (Itaya et al., 1986; Grobe et al., 2010; Hilzendeger et al., 2013). Previous studies have supported the hypothesis that DOCA-salt hypertension is a result of ACE up-regulation in the brain (Itaya et al., 1986) or increase in $AT_1R$ density in some regions of the brain such as subfornical organ, median preoptic nucleus, area prostrema and paraventricular nucleus (Nishimura et al., 1999; Palkovits, Záborszky, 1977; Mangiapane, Simpson, 1980; Casto, 1984; Gutkind, 1988; Grobe et al., 2011). The RAS overactivity associated with $AT_1R$ increase in the brain lead us to point out oxidative stress as a key mechanism in Ang-II-mediated neurogenic hypertension (Kitiyakara and Wilcox, 1998; Houston, 2005; Burmeister et al., 2011; Queiroz et al., 2013). Increased oxidative stress has been found in DOCA-salt hypertension model (Somers et al., 200, Beswick et al., 2001a). The 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 (Beswick et al., 2001a and b), contributing to end-organ damage (Meyer et al., 1993; Beswick et al., 2001b). In the current study we demonstrated that DOCA-salt hypertensive mice presented a greater increase in MAP and that either LA or a 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 (Chen et al., 2001; Landmesser et al., 2003; Braga, 2010; Botelho-Ono et al., 2011; Guimaraes et al., 2012; Queiroz et al., 2012). Our previous study showed that LA reduced hypertension in 2K1C rats (Queiroz et al., 2012). Besides, Takaoka et al (2001) demonstrated that LA was able to decrease systolic BP of DOCA-salt hypertensive rats in a dose considerably higher compared to the one employed in our study.

We previously demonstrated that spontaneously baroreflex sensitivity and sympathetic activity are impaired in DOCA-salt hypertension (Xia et al., 2013). Nevertheless the involvement of antioxidant therapy on these parameters is the first time. In our study, LA treatment improved impaired cardiac sympathetic and vagal tone without affecting vascular sympathetic drive. In addition, the LA increased the SBRS in DOCA-salt hypertensive animals. Moreover, our laboratory previously showed that improvement of autonomic function is associated with ACE2-mediated reduction of oxidative stress in the central

nervous system (Xia et al., 2011). Other study demonstrated that ACE2 gene therapy can restore baroreflex and autonomic functions and prevent the development of hypertension (Feng et al., 2010). Additionally, we documented that knockdown of ADAM17 prevents the reduction of ACE2 levels in the brain is related to an inhibition of DOCA-salt hypertension, thereby ACE2 shedding contributes to development of neurogenic hypertension (Xia et al., 2013).

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 Xia et al. (2013): however, both activities were restored by antioxidant treatment with LA. A previous study showed that ACE2 overexpression in hypothalamus, more precisely in the PVN, increased ACE2 activity and downregulated the $AT_1R$ expression contributing to reduction of Ang-II-mediated hypertension (Sriramula et al., 2011). Ang-II mediates the ADAM17 activation and ACE2 shedding through $AT_1R$ acting as a positive feedback mechanism in the RAS. ACE2/Ang-(1-7)/MasR pathway is the main negative regulator of ACE/Ang-II/$AT_1R$ effects (Patel et al., 2014). Recent data from our group showed, by the first time, that elevated Ang-II levels induce a reduction in ACE2 expression/activity and ACE2 internalization by stimulation of lysosomal degradation via an $AT_1R$-dependent mechanism in neurons (Deshotels et al., 2014). Therefore, oxidative stress from Ang-II-mediated Nox can induce an upregulation of ADAM17, which in turn induces ACE2-shedding and drive the feed-forward mechanism of hypertension. These responses corroborate the idea that both antioxidant therapy as well as an increase in ACE2 expression counteract to ROS formation in the brain and are useful to prevent neurogenic hypertension. The impaired regulation of ACE2 and ADAM17 found in pathological conditions, such as in DOCA-salt hypertension allows the uncoupling of ACE2/Ang-(1-7)/MasR pathway and increase of soluble ACE2, isoform involved in many cardiovascular diseases (Epelman et al., 2008; Epelman et al., 2009).

A recent study has shown that ADAM17 activation induces oxidative stress via up-regulation of Nox4 protein expression and increased Nox activity leading to extracellular matrix accumulation in kidney (Ford et al, 2013). In contrast, another study demonstrated that Nox4 and ROS promote increase in ADAM17 expression by Ang-II-mediated EGFR activation to induce cardiac hypertrophy (Zeng et al, 2013). Studies have acknowledged that Nox2 seems to be more prominent mediator of the injurious effects of Ang-II in the central nervous system during hypertension (Chrissobolis et al., 2012). Injections of adenoviral vectors encoding siRNA targeting Nox2 or Nox4, used to knockdown Nox2 and Nox4 expression in the PVN showed that both

attenuated the development of aldosterone/NaCl-mediated hypertension (Xue et al., 2012). Of note, the current study showed that either Nox2 as Nox4 are up-regulated in hypothalamus of DOCA-salt hypertension mice. In addition the 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, promoting neurogenic hypertension.

The mechanisms by which LA acts to promote the effects presented in this study need to be more investigated. However we already know that LA regenerates endogenous antioxidants (vitamins C and E) and has a property to scavenger free radicals without itself mediates this process (Biewenga et al., 1997). LA is widely known to reduce the expression of metalloproteinase-9 through the inhibition of NF-kB (Packer et al., 1995, Kim et al., 2007), this effect is important to regulate an inflammatory response induced by ROS. Other studies demonstrated that LA improves endothelial NO synthesis (Shay et al., 2008, Shay et al., 2009). Thereby, all these mentioned responses could regulate ROS formation and ameliorate 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. In addition ADAM17 could be a new target to prevent the neurogenic hypertension.

## Perspectives

Our new findings indicate that lipoic acid operates as a novel ADAM17 inhibitor as well as capable to decrease DOCA-salt hypertension mediated by an antioxidant effect. This is supported by the following evidence: (1) ADAM17 protein expression and ADAM17 activity was reduced after treatment using LA in Neuro2A cells; (2) LA was effective in preventing ADAM17-mediated ROS formation in neurons; (3) the beneficial effect induced by LA on MAP and autonomic function are mediated, at least in part, by an improvement in ADAM17 and ACE2 activities; and (4) LA induced an antioxidant effect in hypothalamus of DOCA-salt hypertensive mice. The present study has established the potential effect of the feed-forward mechanism between ADAM17 and oxidative stress and the inhibition of this cycle by LA. Recent studies have been shown that ADAM17 could be activated by ROS, some MAP kinases such as p38, ERK1/2 and PKC, and nitric oxide (Brill et al., 2009; Zeng et al., 2013; Kuan et al., 2013, Chanthaphavong et al., 2012). After ADAM17 activation, this protein can induce the shedding of ACE2, leading to ACE2 secretion and reduction in Ang-(1-7) formation. However the inhibition of some ADAM17 activators is valuable to preserve the ACE2 compensatory effect and prevent the

neurogenic hypertension. Thereby LA has a great value in advance of the understanding of mechanisms underlying in the ROS/ADAM17/ACE2 pathway as well as a novel therapeutic approach in the treatment of hypertension.

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**Disclosures**

None.

**What is New?**

- ADAM17 protein expression and activity were reduced by antioxidant treatment in Neuro2A cells.
- ADAM17 overexpression increased ROS production and this effect was prevented in neurons treated with LA.
- DOCA-salt hypertension was reduced after 21 days-treatment with tempol and LA. Furthermore the autonomic function as well as baroreflex sensitivity was improved in animals treated with LA.
- Failure in ACE2 and ADAM17 activities in hypothalamus of DOCA-salt hypertensive animals were abolished after LA treatment.
- Overexpression in NADPH subunits (Nox2 and Nox4) of DOCA-salt hypertensive mice were blunted in animals treated with LA.

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**FIGURES**

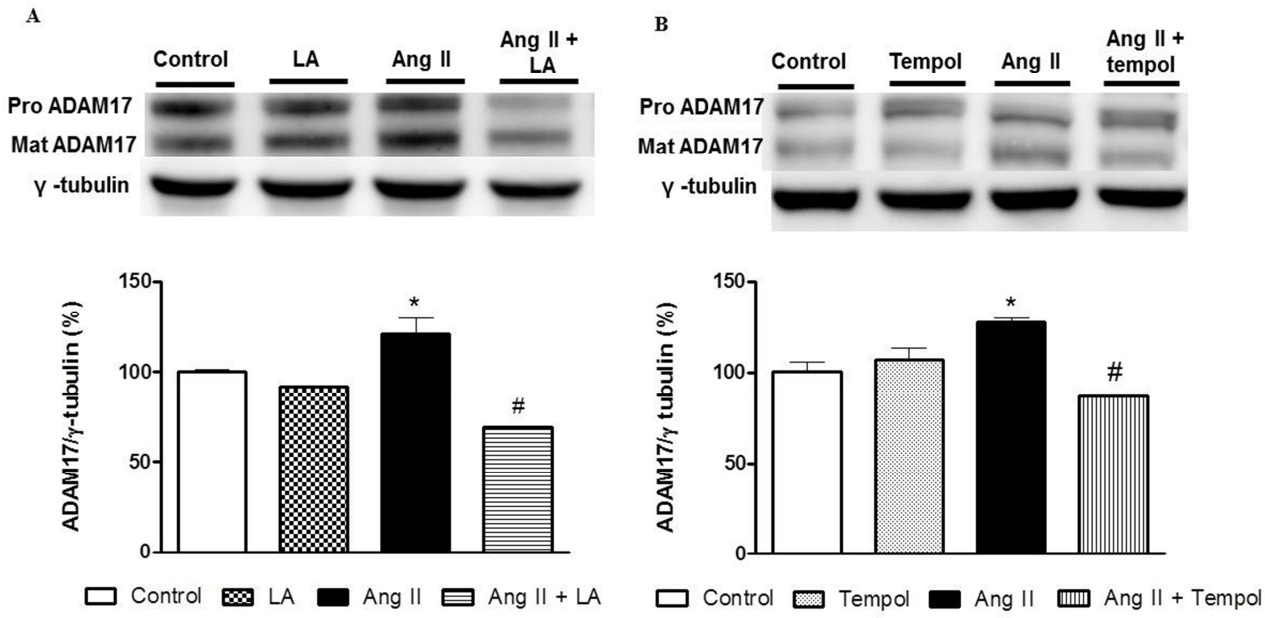


**Figure 1.** ADAM17 expression induced by angiotensin II (Ang-II) in Neuro2A cells. Representative Western Blot gels (Pro and Mat ADAM17) and group data (Mat ADAM17) showing the reduction of the ADAM17expression induced by (**A**) lipoic acid (LA) or (**B**) Tempol. *p<0.05 *vs.* control, and # p <0.05 *vs.* Ang-II. All data are mean ±SEM.

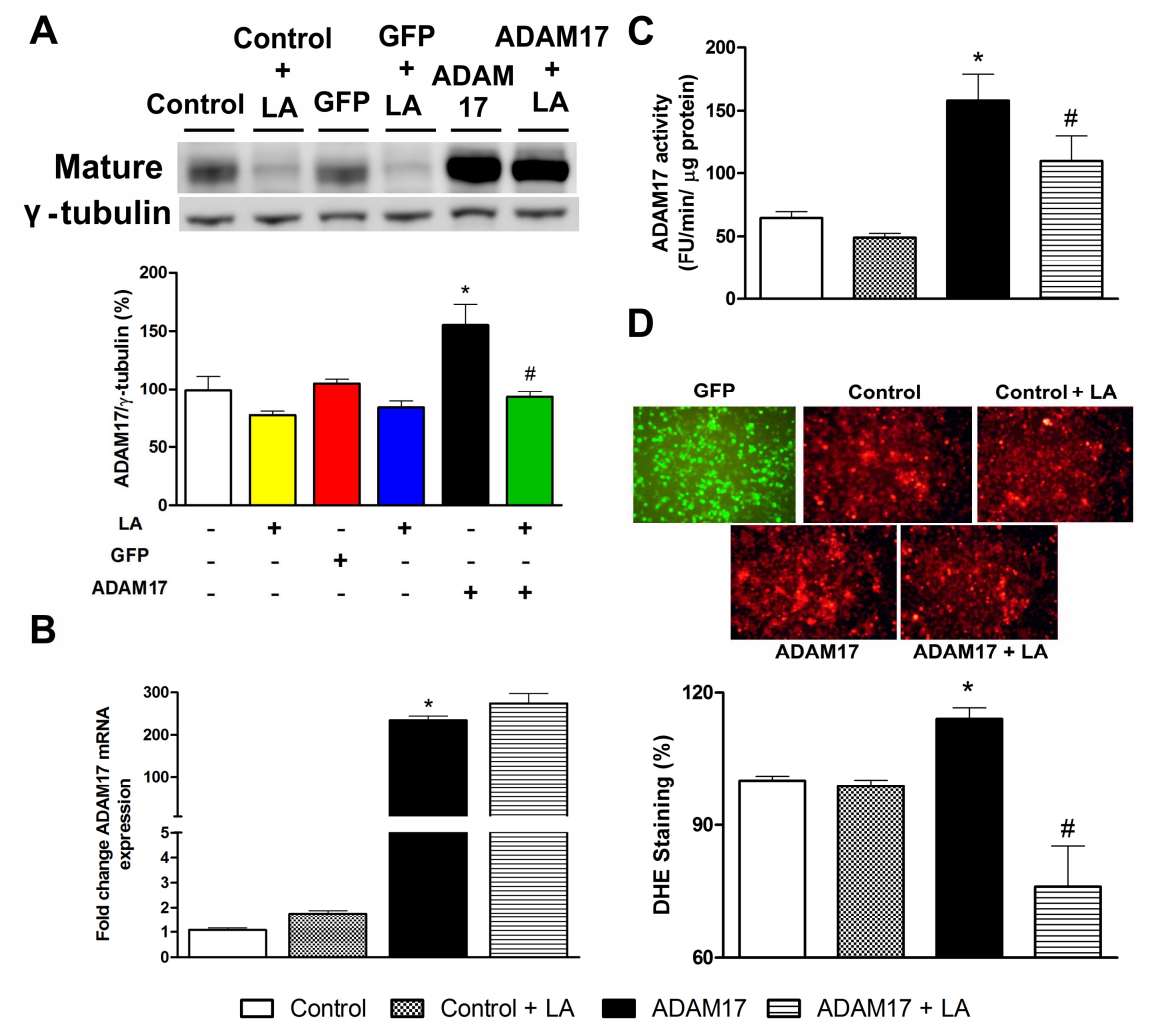


**Figure 2.** Effect LA on ADAM17 protein overexpression induced by ADAM17 plasmid in Neuro2A cells. Western Blot gel representative and group data showing the reduction in overexpression of ADAM17 induced by lipoic acid (LA) (**A**). RT-PCR showed no changes in ADAM17 expression after LA treatment (**B**). ADAM17 activity is reduced by LA treatment (**C**). Representative DHE stained sections and group data showing that ADAM17 overexpression significantly increased ROS production in Neuro2A cells and that LA reduces ROS accumulation (**D**). (GFP: Green fluorescent protein; SiRNA: RNA silencing of ADAM17 protein), *p<0.05 *vs.* Control and #p<0.05 *vs.* ADAM17. All data are mean ±SEM.

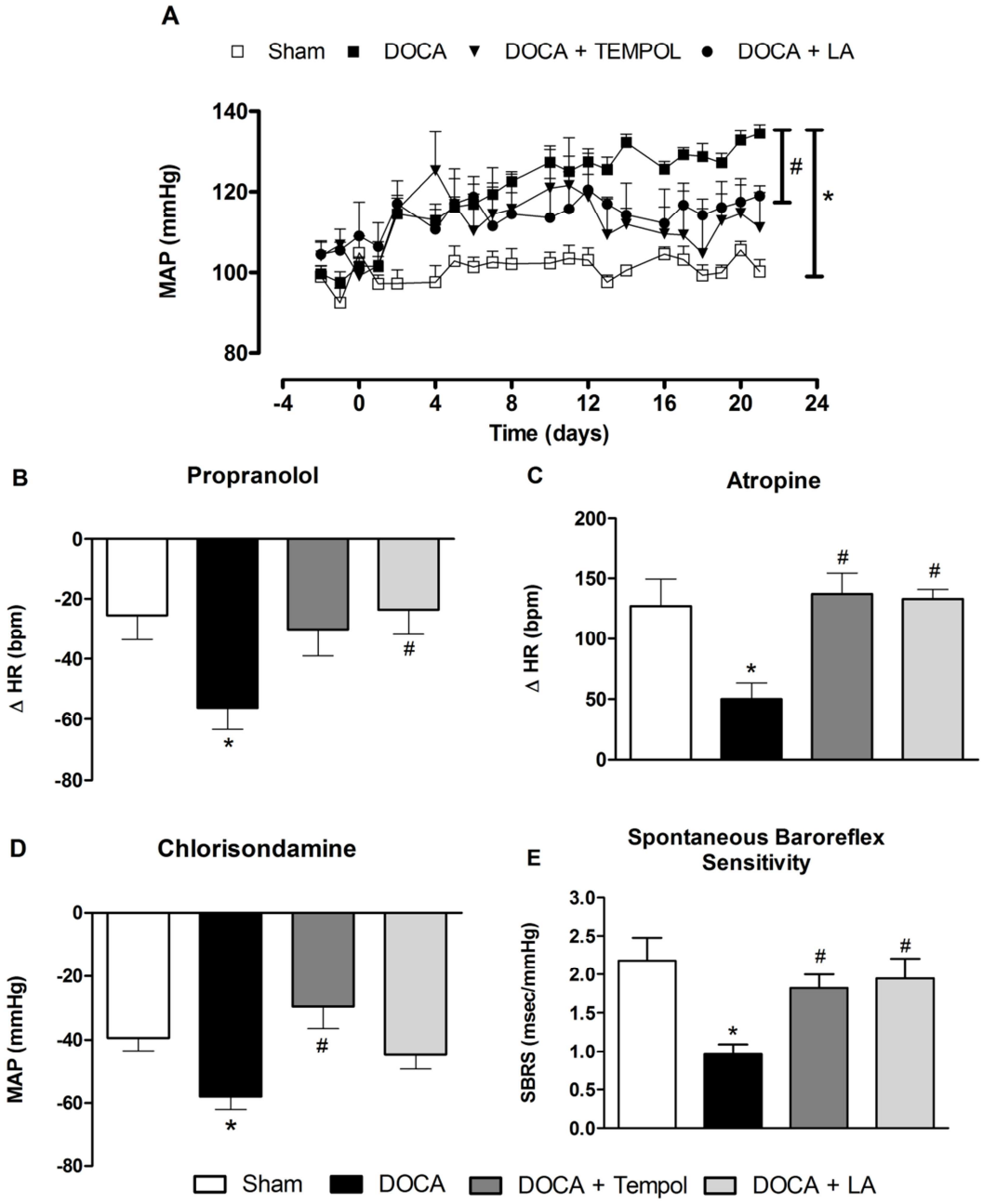


**Figure 3.** Lipoic acid (LA) prevents DOCA-salt-induced hypertension in mice (**A**). Lipoic acid improves impaired cardiac sympathetic (**B**) and vagal tone (**C**) in DOCA-salt-hypertension without affecting vascular sympathetic drive (**D).** Lipoic acid improves baroreflex sensitivity in DOCA-salt-induced hypertensive mice (**E**). *p<0.05 *vs.* Sham and #p<0.05 *vs.* DOCA. All data are mean ±SEM.

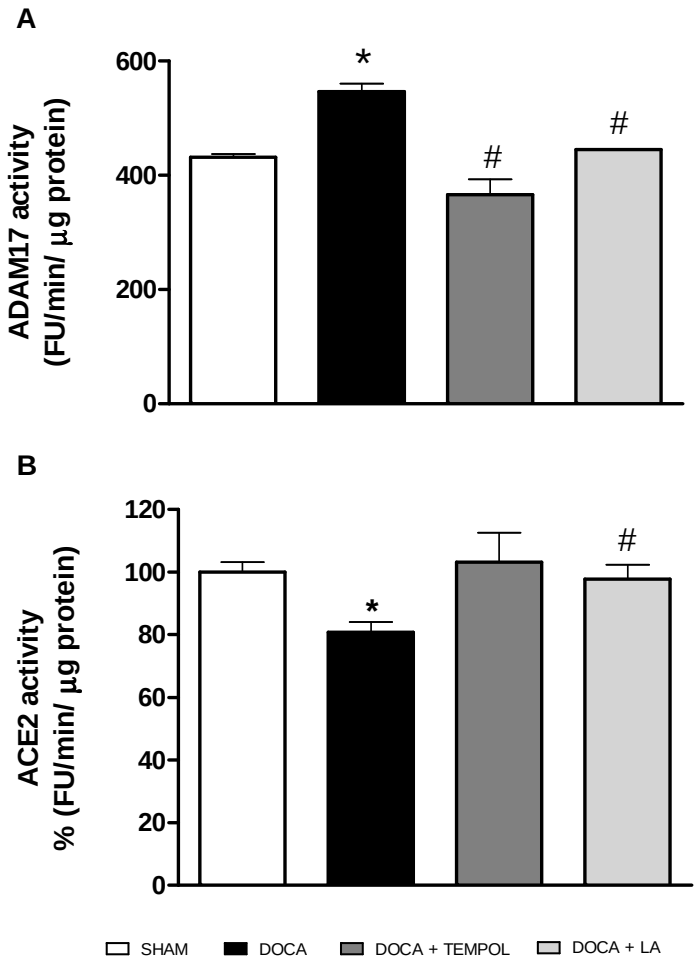


**Figure 4.** ADAM17 and ACE2 activities in the hypothalamus of DOCA-salt hypertensive mice. The increase of hypothalamic ADAM17 activity (**A**) and the decrease of ACE2 activity (**B**) were reduced by lipoic acid treatment in DOCA-salt hypertension mice. *p<0.05 *vs.* Sham and #p<0.05 *vs.* DOCA. All data are mean ±SEM.

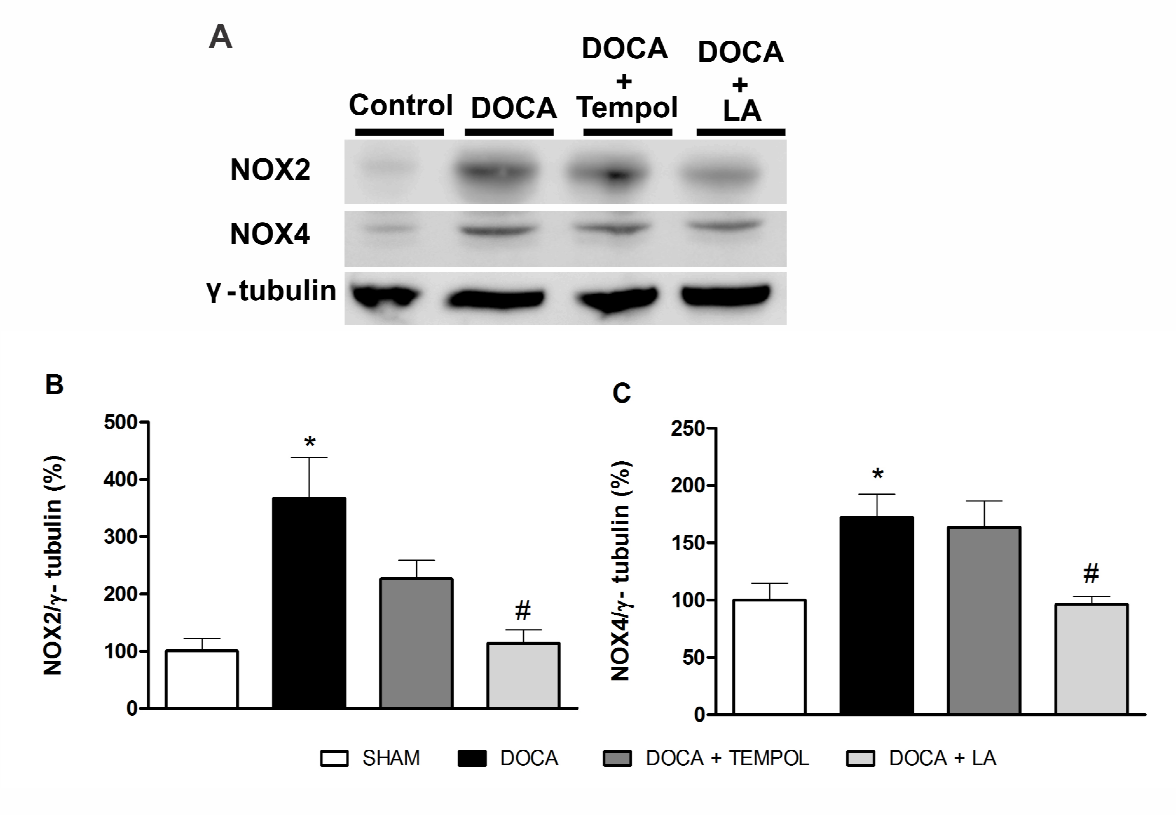


**Figure 5.** Evaluation of hypothalamic NADPH oxidase expression in DOCA-salt hypertensive mice. Representative Western Blot gel and group data showing that lipoic acid reduces the increase in NOX2 (**A**) and Nox4 (**B**) protein expression in the hypothalamus of DOCA-salt hypertensive mice. *p<0.05 *vs.* Sham and #p<0.05 *vs.* DOCA. All data are mean ±SEM.

**ONLINE SUPLEMENT**

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

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Short title: ADAM17 and Oxidative Stress in Hypertension

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**Methods**

Animals

Experiments were performed in adult (6 to 9 weeks of age; 25 to 30 g) C57BL/6J mice. Animals were fed with standard mouse chow and water *ad libitum*. The experimental protocols were reviewed and 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

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®, Clear$H_2O$) 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 (Feng et al., 2010). 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) (Xia et al., 2009). Each injection was separated by a 3-hour recovery period. 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; GIBCOH, 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%, FBS; GIBCOH) at 37 ºC under a humidified 95% and 5% (v/v) mixture of air and $CO_2$. 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 $10^5$ cells/chamber for dihydroethidium (DHE) fluorescence measurement or 10 cm dishes at a density of $10^6$ cells/dish for western blot, ADAM17 assay and RT-PCR experiments.

In other set of experiments, Neuro2A cells were transfected with ADAM17 siRNA and control siRNA using DharmaFECT transfection reagents (Thermo Scientific), according to the manufacturer's instructions. For ADAM17 overexpression, cells were treated with ADAM17 plasmid (1.25 µg/ml, Addgene plasmid 19141) (Lemieux et al., 2007) or GFP plasmid (1.25 µg/ml) as a control using 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. After the cells were harvested for western blotting analysis, DHE staining, ADAM17 activity assay and RT-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) $NaN_3$, pH 7.4) containing a protease inhibitors cocktail (Sigma, St Louis, MO). The lysate was centrifuged twice at 20,800 x *g* at 4ºC for 15 min and the supernatant transferred to a clean tube. Protein concentration was measured using a BCA assay kit (Pierce, Rockford, IL). Cell lysates were mixed with SDS-PAGE sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue), heated at 95ºC for 5 min and loaded onto a 4–15% SDS–polyacrylamide gel for electrophoresis (Life Technologies, Carlsbad, CA). Proteins were transferred to nitrocellulose membrane at 200 mA for 2h by semi-dry blot (Fisher Scientific, Houston, TX). Membranes were blocked with 5% non-fat milk in PBST (1.47 mM $NaH_2PO_4$, 8.09 mM $Na_2HPO_4$, 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, ab 2051, 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) or goat anti-mouse IgG-HRP 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 (Abcam, ab7291; 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.

Protein extracted from hypothalamus (20 µg) was processed for Western blotting as described previously (Feng et al. 2010). After euthanasia, brains were quickly removed from the animals and immediately frozen on dry ice. Total protein was extracted from

hypothalamus 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, ab 2051, 1:1000); anti-Nox4 [UOTR1B492] (Abcam, ab 109225, 1:1000) and mouse anti-gp91$^{phox}$ (BD Transduction Laboratories®, cat 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 third day posttransfection, 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 described previously (Pedersen et al., 2011). Tissue from brain hypothalamus was homogenized with ACE2 activity reaction buffer (in mmol/L: NaCl: 1000, Tris: 75, ZnCl2: 0.5, pH 7.5) and centrifuged at 20,800 x g for 10 min and the supernatant transferred to a clean tube. Proteins concentration was measured using a BCA assay kit (Pearce, Rockford, IL). 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 VI (FPSVI, 7Mca-Y-V-A-D-A-P-K(Dnp)-OH) (R&D systems, Minneapolis, MN). This substrate contains a C-terminal dinitrophenyl moiety that quenches the inherent fluorescence of the 7-methoxycoumarin group by resonance energy transfer. ACE2 removes the C-terminal dinitrophenyl moiety in the FPSVI thus increasing fluorescence emission at 405 nm under excitation at 320 nm. Tissue lysates containing 100 µg of protein were incubated with FPSVI (100 µmol/L) and captopril (10 µmol/L) in reaction buffer (100 µL) at 37°C. Non-specific enzyme activity was measured by including DX600 (1µmol/L), a

specific ACE2 inhibitor, (Phoenix Pharmaceutical, Belmont, CA). Fluorescence emission was monitored using a SpectraMax M2 Fluorescence Reader (Molecular Devices, Sunnyvale, CA). The noise to signal ratio for enzyme activity in the absence of substrate or cell extract was <5%. Specific ACE2 activity was calculated by subtracting the total activity in the presence of captopril (10 μmol/L), from the activity in the presence of both captopril (10 μmol/L) and DX600 (1 μmol/L). Data are presented as amounts of substrate FPSVI converted to product per minute, normalized for total protein and expressed as arbitrary fluorescence units.

Reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from Neuro2A cells 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). Primer sequences used for ADAM17 real time PCR are Forward: 5'-CGT GGT TGG TGA GCC TGA CT-3' and Reverse: 5'-TTA TAT TCT GCC CCA TCT GTG TTG-3'. Data were analyzed by the ΔΔCT 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 $P<0.05$.

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# Considerações e Perspectivas

## 4 CONSIDERAÇÕES FINAIS E PERSPECTIVAS

No presente estudo foram utilizados dois diferentes modelos de hipertensão arterial: os modelos dois-rins-um-clipe (2R1C) e DOCA-sal.

Com o intuito de induzir a hipertensão renovascular do tipo 2R1C, foi utilizada a técnica descrita por Goldblatt et al. (1934) e adaptada por Shaffenburg (1959) para animais de pequeno porte. Este modelo de hipertensão é induzido cirurgicamente pela constrição da artéria renal, induzindo a isquemia renal e, consequentemente, ativando um dos sistemas vasoconstritores mais potentes do organismo, que é o sistema renina angiotensina (Prieto-Carrasquero et al., 2008).

DOCA-sal é um modelo de hipertensão com baixos níveis de renina, onde há a administração de um mineralocorticoide, desoxicorticosterona, em animais uni-nefrectomizados e com suplementação de 1% de NaCl. Goodwin et al. (1969), demostraram que é necessário o tratamento conjunto do excesso diário de sal e do esteroide para o desenvolvimento da hipertensão DOCA-sal, já que somente o tratamento com DOCA ou apenas o consumo excessivo de sal não são capazes de proporcionar uma elevação da pressão arterial. Nesse tipo de hipertensão há uma supressão do SRA periférico, porém há uma supra regulação do SRA à nível do SNC (Grobe et al., 2011).

Apesar das diferenças apresentadas nos modelos de hipertensão estudados neste trabalho, e das vantagens e desvantagens que são inerentes a cada modelo, ambos possuem, pelo menos, um fator em comum que é o aumento dos níveis de Ang II. Além disso, são de fácil execução e muito eficientes no estudo da hipertensão arterial.

Durante a infusão periférica de Ang II, bem como no modelo de hipertensão DOCA-sal, foi observado que citocinas pró-inflamatórias, incluindo TNF-α, IL-1β, IL-6 e a quimiocina MCP-1 apresentavam-se elevadas no núcleo paraventricular do hipotálamo (Lu et al., 2009; Sun et al., 2004; Sriramula et al., 2008, 2011; Xia et al., 2013). Esses estudos corroboram a hipótese da hipertensão arterial como uma condição inflamatória. Devido ao interessante papel dos fatores imunológicos na regulação da hipertensão arterial, bem como da participação do SNC no controle da pressão arterial, essas hipóteses ressaltam a importância da conexão neuro-imune-fisiológica no processo da hipertensão arterial.

A Ang II induz seus efeitos sobre a pressão arterial, por uma transativação do EGFR, mediada por receptores AT1 (Suzuki et al, 2005;. Saito et al, 2001; Ohtsu et al, 2006). A Ang II ao se ligar ao receptor AT1, que é acoplado à proteína Gq/11, ativa a fosfolipase C do tipo β e assim aumenta a concentração de $Ca^{2+}$ citosólico, que por sua vez, favorece a estimulação da proteína cinase C (PKC) (Higuchi et al., 2007). Esta cinase, juntamente com ROS e elevações nas concentrações livre de $Ca^{2+}$ podem induzir a transativação do EGFR (Ohtsu et al., 2006), a qual requer a clivagem do precursor ligante de EGFR ancorado à membrana, induzida por ADAM17 (Oikawa et al., 2014).

Os mecanismos de ativação da ADAM17 ainda permanecem controversos, entretanto, algumas evidências apontam como possíveis ativadores as ROS, NOX-4 e as proteínas da família das MAP-cinases (Brill et al., 2009; Zeng et al., 2013; Kuan et al., 2013). Recentemente o óxido nítrico (NO) foi revelado como um ativador de TACE. Chanthaphavong et al. (2012)

demostraram que fatores estimuladores da sintase do óxido nítrico (iNOS), como o LPS, por meio do receptor *Toll-like* 4 induz a transcrição da iNOS, que culmina na produção de NO. Esta molécula lipossolúvel liga-se a sintase de guanilil solúvel (sGC) que promove a formação do nucleotídeo cíclico cGMP e ativação de PKG, que por sua vez promove a fosforilação e ativação da ADAM17.

O NO também possui um papel importante na ativação da ADAM17. Estudos de Dada e Sznajder (2011) mostraram que elevações nas concentrações de $Ca^{2+}$ também podem induzir a ativação da NOS e promover a liberação de NO, o qual tem a capacidade de inibir a enzima citocromo oxidase da cadeia transportadora de elétrons e por fim leva a formação de ROS, ativando a ADAM17.

Após a ativação por diversos fatores citados, a ADAM17 é conhecida por promover *shedding* sobre a enzima ECA2, importante enzima do SRA. Em caso de *shedding*, a ECA2 se torna solúvel e deixa de converter Ang II em Ang 1-7. Assim o efeito compensatório desta enzima está comprometido, bem como os efeitos benéficos promovidos pela ligação da Ang 1-7 ao seu receptor Mas. Entretanto, Lambert et al. (2008) sugeriram que a ligação da proteína calmodulina (CaM) à cauda citoplasmática da ECA2 seria um mecanismo de proteção contra o *shedding* da ECA2.

No presente trabalho foi observado que o ácido α-lipóico, um antioxidante que vem sendo bastante estudado, promoveu um efeito hipotensor em animais com hipertensão renovascular. Este efeito foi acompanhado de um aumento da sensibilidade do barorreflexo após 14 dias de tratamento em ratos 2R1C.

Em colaboração com o laboratório do professor Eric Lazartigues (LSUHSC/New Orleans, EUA), investigou-se o papel do ácido lipóico em abordagens *in vitro* e *in vivo*. Para os experimentos *in vitro,* utilizamos células Neuro2A, e foi verificado que o antioxidante em estudo foi capaz de promover uma redução na expressão da ADAM17 à níveis basais, como também em células que superexpressaram ADAM17. O ácido lipóico também diminuiu a atividade da ADAM17 com redução da produção de ROS em células Neuro2A superexpressas com ADAM17. Já para os estudos *in vivo*, foi utilizado o modelo de hipertensão DOCA-sal em camundongos. Neste modelo foi observado que o ácido lipóico reduz a pressão arterial de animais DOCA-sal, melhora da sensibilidade do barorreflexo espontâneo e a função autonômica. Após a eutanásia dos animais, os cérebros foram removidos e estudados. Nas análises utilizando o hipotálamo isolado, demonstrou-se que o ácido lipóico preveniu a redução da atividade da enzima ECA2, bem como o aumento da atividade da ADAM17. Além disso, o antioxidante atenuou a superexpressão da enzima NADPH oxidase em camundongos hipertensos DOCA-sal.

A importância da avaliação dos efeitos no hipotálamo deve-se ao fundamental papel desta área do SNC no controle da hipertensão arterial mediado pelo estresse oxidativo e Ang II. Por exemplo, o reflexo simpático cardíaco mostrou-se estar envolvido na formação de ROS mediada por Ang II no núcleo paraventricular do hipotálamo (Zhang et al., 2006). Além disso, efeitos pressóricos centrais e sistêmicos induzidos por Ang II estão conectados com a formação de ROS mediada por NOX no PVN (Erdos et al., 2006; Kang et al., 2009; Burmeister et al., 2011).

Em resumo, nossos dados sugerem que o ácido lipóico induz seus efeitos sobre a pressão arterial de ratos 2R1C e de camundongos DOCA-sal, por quebrar o ciclo de retroalimentação positiva entre ADAM17 e estresse oxidativo, e assim preservar a atividade compensatória da ECA2. Além disso, podemos sugerir que a ADAM17 constitui-se de um novo alvo terapêutico na prevenção e tratamento da hipertensão arterial.

Diante dos diversos mecanismos pelos quais a proteína ADAM17 pode ser ativada, somado ao fato de que neste estudo o AL promoveu a regulação desta proteína por um mecanismo antioxidante, torna-se disponível um composto que pode servir como um futuro fármaco no tratamento da hipertensão, ou até mesmo uma nova ferramenta farmacológica em estudos experimentais. Entretanto, estudos adicionais necessitam ser realizados para uma melhor avaliação e pesquisa deste antioxidante, além de outras abordagens para o aprofundamento dos possíveis mecanismos de ação desta droga.

Cabe ressaltar que este trabalho é pioneiro em documentar que a ECA2 e ADAM17 participam do mecanismo promovido pelo ácido lipóico. Ademais, produzimos dados relevantes sobre possíveis fatores envolvidos na regulação destas proteínas. Para estudos futuros, sugere-se a avaliação de outros fatores ativadores da ADAM17, que incluem: proteínas cinases, NO, caveolina-1, entre outros. Em relação a ECA2, podemos analisar a participação do $Ca^{2+}$ e da proteína CaM na prevenção do *shedding* por ADAM17, já que a ADAM17 é a única metaloproteinase que é descrita como sendo capaz de clivar a ECA2.

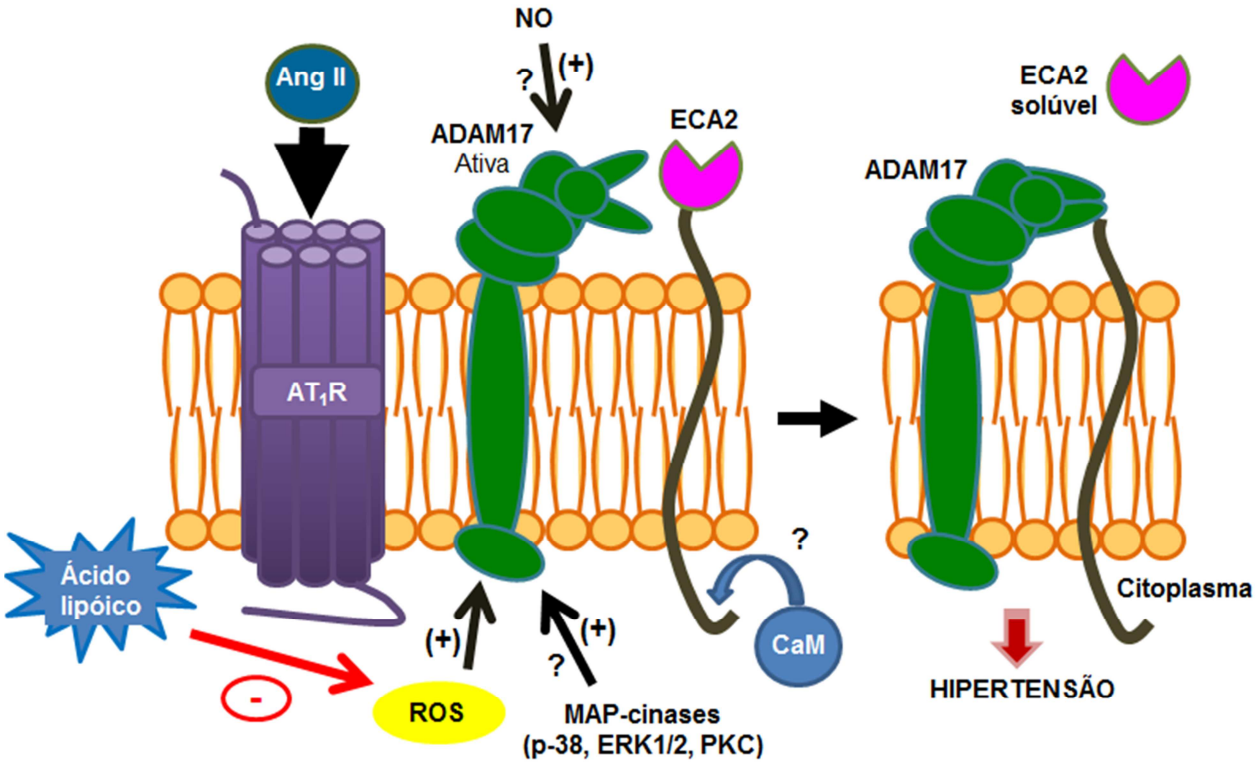


**Figura 3**. **Modelo representativo da ação do ácido lipóico sobre a regulação da proteína ADAM17 e ECA2**. No caso de um aumento na atividade do SRA, Ang II se liga ao seu receptor AT1, promovendo uma ativação da NADPH oxidase e posterior formação de ROS. Estas espécies podem induzir a ativação da ADAM17, bem como outros candidatos, como, por exemplo: proteínas da família MAP-cinase, NO, entre outros. Uma vez a ADAM17 no seu estado ativo, irá funcionar como uma *sheddase* e promover a clivagem de proteínas membranares, como a ECA2. Esta enzima em seu estado solúvel não irá favorecer a formação de Ang 1-7 e seu efeito compensatório será interrompido, favorecendo a hipertensão. Entretanto, o ácido lipóico reduz a produção de ROS, dimunuindo a expressão e atividade da ADAM17, favorecendo a ação anti-hipertensiva da ECA2. (Ang II: angiotensina II; ROS: espécies reativas de oxigênio; ATIR: receptor para angiotensina tipo 2; ECA2: enzima conversora de Angiotensina tipo 2; ADAM17: desintegrina e mataloprotease 17; CaM: calmodulina).

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# Anexos

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August 22, 2014

Dr. Valdir de Andrade Braga
Federal University of Paraiba
Joao Pessoa,
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Dear Valdir,

It is with great pleasure that I write this report concerning Thyago Moreira de Queiroz' 1-year visit in my laboratory. As you know, I have been very pleased with Thyago's performance over the last year and I am confident that the data he generated will lead to a strong publication.

As you know, we previously reported that ACE2 (Angiotensin converting enzyme type 2) compensatory activity is impaired by A Disintegrin And Metalloprotease 17 (ADAM17) in neurogenic hypertension and that lack of ACE2 is associated with oxidative stress. To clarify the relation between ADAM17 and oxidative stress, Thyago used cell culture, biochemistry and *in vivo* approaches. Notably, he showed that ADAM17 expression was associated with an increase in oxidative stress in cell cultures and in hypertensive mice. Moreover, he demonstrated that lipoic acid, an anti-oxidant, could reduce ADAM17 expression and, more importantly, that it could blunt the development of hypertension. Together, these data suggest that lipoic acid might preserve ACE2 compensatory activity by breaking the feed-forward cycle between ADAM17 and oxidative stress. We are planning on submitting these data for publication to the journal *Hypertension* in the coming weeks.

As we originally planned, within my group Thyago received an intensive training in Physiological Genomics, allowing him to link gene to function. He has learned to use molecular techniques such as quantitative real-time RT-PCR, genotyping and cell cultures. He has been involved in, and has himself performed, some physiological experiments such as blood pressure recording, measurement of baroreflex function, analysis of autonomic regulation in combination with various pharmacological treatments.

In addition to this hands-on training, Thyago participated in the activities of my laboratory (lab meetings, journal clubs, analysis of the literature) and the department of Pharmacology (seminars, work-in-progress). Thyago also had the opportunity to attend the Experimental Biology meeting held in San Diego in the Spring of 2014 and recently presented his data at the 1st Pan-American meeting of Physiology held at Iguacu falls in Brazil.

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In closing, I would like to reiterate my satisfaction regarding Thyago's stay in my lab and assure you that he met all my expectations. I believe that his participation in the "CNPq Sandwich program" will have a very positive influence on his scientific career.

Sincerely,

Eric Lazartigues, Ph.D., FAHA
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## PRODUÇÃO CIENTÍFICA DURANTE O DOUTORADO

### Resumos publicados em anais de congressos e apresentação de trabalhos:

- **QUEIROZ, T. M**, MENDES - JUNIOR, L. G., GUIMARÃES, D. D., FRANCA-SILVA, M. S., NALIVAIKO, E., BRAGA, V. A.CARDIORESPIRATORY EFFECTS INDUCED BY 2-NITRATE-1,3-DIBUTHOXYPROPAN ARE REDUCED BY NITRIC OXIDE SCAVENGER IN RATS In: XLVIII Congresso Anual da Sociedade Brasileira de Fisiologia, 2013, Ribeirão Preto - SP.

- **QUEIROZ, T. M**, GUIMARÃES, D. D., MENDES - JUNIOR, L. G., BRAGA, V. A. ANTI-OXIDANT THERAPY WITH ALPHA-LIPOIC ACID REDUCES HYPERTENSION AND IMPROVES DEPRESSED BAROREFLEX SENSITIVITY IN RENOVASCULAR HYPERTENSIVE RATS In: IV Simpósio de Produtos Naturais, 2012, João Pessoa.

- **QUEIROZ, T. M**, MENDES - JUNIOR, L. G., GUIMARÃES, D. D., BRAGA, V. A. Efeito do ácido lipóico sobre a reatividade vascular em ratos com hipertensão renovascular In: VII Reunião Regional da Federação de Sociedades de Biologia Experimental-FeSBE, 2012, Maceió - AL. Anais da VII Reunião da FeSBE. , 2012.

- GUIMARÃES, D. D., **QUEIROZ, T. M**, MENDES - JUNIOR, L. G., BRAGA, V. A. O ácido alfa-lipóico promove efeito anti-hipertensivo e aumento da sensibilidade do barorreflexo em ratos com hipertensão renovascular In: VII Reunião Regional da Federação de Sociedades de Biologia Experimental-FeSBE, 2012, Maceió - AL. Anais da VII Reunião da FeSBE. , 2012.

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- **QUEIROZ, T. M**.; XIA, H. ; BRAGA, V. A. ; LAZARTIGUES, E. ADAM17-mediated increase in oxidative stress is reduced after α-lipoic acid treatment. In: Panamerican Congress of Physiological Sciences, 2014, Foz do Iguaçu. 1st Congress of Panamerican Congress of Physiological Sciences, 2014.

**Premiações**

- Menção honrosa para o trabalho: ADAM17-mediated increase in oxidative stress is reduced after α-lipoic acid treatment. **QUEIROZ, T.M**.; XIA, H.; BRAGA, V. A.; LAZARTIGUES, E. Panamerican Congress of Physiological Sciences, 2014, Foz do Iguaçu. 1st Congress of Panamerican Congress of Physiological Sciences, 2014.

- Menção Honrosa para o trabalho: Nitric Oxide as a target for the hypotensive and vasorelaxing effects induced by (z)-ethyl 12-nitrooxy-octadec-9-enoate in rats. MACHADO, N. T., MACIEL, P. M. P., ALUSTAU, M. C., **QUEIROZ, T. M**, FURTADO, F. F., SILVA, T. A. F., VASCONCELOS, W. P., SANTOS, P. C., ATHAYDE-FILHO, PETRÔNIO F., MEDEIROS, I. A. Na edição 2013 do Prêmio José Ribeiro do Valle, SBFTE em parceria com a Biolab-Sanus Farmacêutica.

- Menção Honrosa - pela apresentação Oral: Anti-oxidant therapy with alpha-lipoic acid reduces hypertension and improves depressed baroreflex sensitivity in renovascular hypertensive rats, IV Simpósio de Produtos Naturais – UFPB, 2012.

**Artigos completos publicados em periódicos**

- **QUEIROZ, THYAGO M**., MENDES-JÚNIOR, LEÔNIDAS G., GUIMARÃES, DRIELLE D., FRANÇA-SILVA, MARIA S., NALIVAIKO, EUGENE, Braga, Valdir A. Cardiorespiratory effects induced by 2-nitrate-1,3-dibuthoxypropan are reduced by nitric oxide scavenger in rats. Autonomic Neuroscience: Basic & Clinical. , v.181, p.31 - 36, 2014.

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