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# SOBRECARGA DE SÓDIO E TRATAMENTO NO PERÍODO PERINATAL COM INIBIDOR DE ENZIMA CONVERSORA DE ANGIOTENSINA OU α-TOCOFEROL ALTERAM A FUNÇÃO RENAL E CARDIOVASCULAR EM RATOS

Orientadora: Profa. Dra. ANA DURCE OLIVEIRA DA PAIXÃO

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Tese apresentada à Universidade Federal de Pernambuco como parte dos requisitos para obtenção do título de Doutor em Bioquímica e Fisiologia.

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**Aprovado por:**

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Com amor e carinho a minha família. Meus pais José Vicente Cabral *(in memoriam) e* Maria Tavares Cabral, e a minha irmã, Edcaroline Tavares Cabral pelo incentivo à minha formação acadêmica.

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**“*O conhecimento nunca está acabado. É uma teia que vamos tecendo a partir da superação dos limites”.* Pe. Fábio de Melo**

## RESUMO

Elevada ingestão de sódio durante o desenvolvimento induz na prole disfunções cardiovasculares e renais. Intervenções durante o período pós-natal podem prevenir tais alterações. Neste trabalho foram investigados se o enalapril, um inibidor da enzima conversora de angiotensina ou o α-tocoferol, administrados por 3 semanas após o desmame previnem alterações programadas pela sobrecarga de sódio pré-natal. Adicionalmente, foi avaliado o efeito do α-tocoferol quando administrado em um período mais precoce, durante a lactação. O protocolo experimental foi dividido da seguinte forma: i) ratos machos adultos Wistar foram obtidos de mães mantidas, durante a gravidez e lactação, com NaCl 0,17 M e ii) ratos machos obtidos de mães mantidas com solução de NaCl 0,3 M, desde 20 dias antes do acasalamento até o parto. A prole de mães que receberam salina 0,17 M durante o período perinatal apresentou nos túbulos proximais: atividade aumentada e maior expressão da subunidade alfa da ($Na^+$-$K^+$)-ATPase, atividade da $Na^+$-ATPase insensível a ouabaína permaneceu inalterada, no entanto, sua resposta à Angiotensina II (AngII) foi perdida. A atividade das proteínas kinases C e A e as substâncias reativas ao ácido tiobarbitúrico (TBARS) apresentaram-se aumentadas, assim como a densidade de colágeno e a expressão de AngII. O tratamento com enalapril reverteu praticamente todas as alterações produzidas pela sobrecarga salina, no entanto, produziu no grupo controle alterações de função renal. A prole de mães que receberam salina 0,3 M durante a gestação apresentou, sobretudo alterações do desenvolvimento cardíaco, as quais foram somadas aos efeitos do α-tocoferol pós-natal. O α-tocoferol produziu elevação da pressão arterial, hiporesponsividade à AngII e atrofia cardíaca, e quando associado ao tratamento pré-natal com salina produziu redução na sensibilidade do baroreflexo. **Conclusão**: A duração do tratamento perinatal, ou seja, somente pré-natal ou pré-natal mais período do aleitamento parecem decisivos na extensão de efeitos sobre a função renal. Por outro lado, o tratamento com enalapril ou α-tocoferol durante o período pós-natal pode reverter alterações produzidas pela sobrecarga de sódio perinatal, no entanto pode programar alterações funcionais cardíaca e renal que são irreversíveis.



Palavras-chave: Sobrecarga de sódio. Desenvolvimento fetal. α-tocoferol. Enalapril. Função renal. Função cardíaca.

## ABSTRACT

Maternal high sodium intake over pregnancy leads to renal and cardiovascular dysfunctions in the offspring. Early interventions could prevent some of these dysfunctions. This work investigated whether the enalapril, an angiotensin converting enzyme inhibitor, or the α-tocopherol, administered for 3 weeks after the weaning, prevent alterations provoked by prenatal sodium overload. Additionally, it was investigated the α-tocopherol effect administered over lactation. The experimental protocol was as it follows: i) Adult male Wistar rats were born from dams provided during pregnancy and lactation with 0.17 M NaCl solution and; ii) adult male rats were born from dams provided 0.3 M NaCl, from 20 days before pregnancy up to parturition. The offspring of dams on 0.17 M NaCl showed in the proximal renal tubules: increased activity of $(Na^+ + K^+)$ATPase, increased expression of the α subunit of this enzyme, unaltered activity of $Na^+$-ATPase, hyporesponsiveness of $Na^+$-ATPase to angiotensin II, increased activity of PKC and PKA and increased levels of tiobarbituric acid reactive substances (TBARS). In the kidney, they showed increased content of collagen and angiotensin II. Enalapril prevented molecular alterations produced by sodium overload; however it changed renal function in control rats. The offspring of mothers that received 0.3M saline over pregnancy exhibited changes in cardiac development that were summed up to postnatally administered α-tocopherol effects. α-Tocopherol led to hyporesponsiveness to angiotensin II-induced hypertension and cardiac atrophy. When it was associated with prenatal treatment with sodium overload, α-tocopherol led to an attenuation in the baroreflex sensitivity. Conclusion: The sodium overload length, it means, only prenatal or prenatal plus postnatal over lactation, seem crucial to the extension of cardiovascular and renal effects. On the other hand, treatment with enalapril or α-tocopherol, at early periods of development, may prevent alterations provoked by sodium overload during perinatal period. However, these substances may programming irreversible changes in renal and cardiac function.



Key-words: Sodium overload. Fetal development. α-tocopherol. Enalapril. Renal function. Cardiac function.

## LISTA DE ABREVIATURAS

AngII- angiotensina II

ATP- trifosfato de adenosina

$Ca^{+2}$- cálcio

$[Ca^{+2}]i$- concentração de cálcio intracelular

CMs- cardiomiócitos

COX-2- ciclo-oxigenase 2

DNA- ácido desoxirribonucléico

ERK- proteína quinase ativada por sinal extracelular

ERONs- espécies reativas de oxigênio e nitrogênio

HIF-1- fator induzível por hipóxia

IECA- inibidor da enzima conversora de angiotensina

JNK- quinase N-terminal c-jun

MAPK- proteína quinase ativada por mitogénos

$Na^{+}$- sódio

NaCl- cloreto de sódio

NADPH- nicotinamida adenina dinucleotídeo fosfato

PAS- pressão arterial sistólica

PMCA- $Ca^{+2}$-ATPase de membrana plasmática

PKC- proteína quinase C

RNA- ácido ribonucléico

SERCA- $Ca^{+2}$-ATPase de retículo sarco/endoplasmático

SHR- ratos espontaneamente hipertensos

SRAA- sistema renina angiotensina aldosterona

α-TPP- proteína de transferência do α-tocoferol

VEGF- fator de crescimento derivado do endotélio

**SUMÁRIO**

## 1. INTRODUÇÃO

Nossos ancestrais tinham como hábito alimentar uma baixa ingestão de sódio uma vez que as fontes de alimento adivinham do extrativismo e da caça, que naturalmente tem como características o baixo teor de sódio. Além disso, eles não adicionavam sal aos alimentos consumidos (BLACKBURN & PRINEAS, 1983; EATON & KONNER 1985; MENETON *et al*, 2005). Pressupõe-se que a humanidade traz a herança genética (MENETON *et al*, 2005), que por sua vez influencia os sistemas fisiológicos e os mecanismos regulatórios, principalmente o sistema cardiovascular, a estarem adaptados ao baixo teor de sódio consumido na dieta. Desta forma, para evitar a perda excessiva de sódio do organismo, a ativação do sistema renina angiotensina aldosterona (SRAA) é requerida bem como a ativação do sistema nervoso simpático que favorecem à reabsorção de sódio pelos rins, e desta forma mantêm o balanço hidroeletrolítico e os níveis pressóricos adequados (TAKAHASHI *et al*., 2011).

O consumo de sódio entre as populações é bastante variado e parece estar relacionado aos hábitos culturais. Há uma correlação positiva entre o teor de sódio ingerido e os níveis de pressão arterial sistólica (PAS). Algumas civilizações têm como hábito alimentar a ingestão reduzida de $Na^+$ e isto tem sido correlacionado com os níveis de PAS mais baixos quando comparados às populações que adicionam sal de forma exacerbada aos alimentos (JOOSENS, 1980). O estudo INTERSALT compreende um levantamento epidemiológico internacional que correlaciona a PAS com a ingestão de sódio. A ingestão de sódio é estipulada pela sua excreção urinária. Este levantamento foi realizado em algumas tribos indígenas existentes na Amazônia Brasileira, como por exemplo a tribo Yanomami, e foi observada uma correlação positiva entre os níveis de PAS e o consumo de sódio. Além disso, não foi observado nessa população um aumento da PAS com a idade (MANCILHA-CARVALHO & SOUZA E

SILVA, 2003). Alguns estudos em que foram avaliados outras tribos indígenas brasileiras como a Amondava (PAVAN *et al.*, 1999), a Suruí (FLEMING-MORAN *et al*., 1991) e a Guarani (MEYERFREUND *et al*., 2009) também foram observados níveis mais baixos de PAS, embora, nesta última população, esta característica parece não estar associada a uma baixa ingestão de sódio na dieta.

Em populações em que a ingestão de sódio é menor que 3 g/dia o número de indivíduos hipertensos é menor quando comparada às populações em que a ingestão de sódio alcança valores próximos a 30 g/dia. Além disso, nessas populações a alta ingestão de sódio tem uma relação direta com a PAS e com a idade, ou seja, quanto maior o consumo de sódio maiores são os níveis de PAS, a qual é aumentada com a idade (MENETON *et al*, 2005). Diferente do que foi observado em tribos indígenas no Brasil, uma tribo situada no Iran, a Quash'Qai, consome uma quantidade de sódio semelhante às populações urbanas e a PAS aumenta com a idade (PAGE *et al*., 1981). O mesmo foi observado em uma população da Caxemira do Norte que costuma tomar chá com adição de sal (MIR & NEWCOMBE, 1988).

## 2. Papel do sódio nos sistemas fisiológicos

O íon sódio ($Na^+$) é o eletrólito predominante do fluído extracelular (plasma sanguíneo e líquido intersticial), e juntamente com o cloreto e o bicarbonato ($HCO_3^-$), constituem os principais íons desse compartimento. Por outro lado, a sua concentração no meio intracelular apresenta-se menor quando comparado ao meio extracelular e ao íon potássio ($K^+$) e este fato deve-se a função da bomba eletrogênica ($Na^+$-$K^+$)ATPase presente em todas as células dos organismos vivos. Esta diferença de concentração entre os meios intra e extracelular é essencial para a manutenção das funções celulares.

Outra importante função do $Na^+$ que está associada ao seu gradiente de concentração através da membrana plasmática, é a de auxiliar a condução do impulso elétrico e a modulação da força contrátil do músculo cardíaco (SARAH *et al*, 2005), bem como, da musculatura lisa vascular (BLAUSTEIN, 1977). No contexto da contração muscular, o íon $Na^+$ apresenta um papel importante na manutenção do gradiente de cálcio ($Ca^{2+}$) através da membrana e dessa forma regula o estiramento das fibras cardíacas e o tônus vascular durante o relaxamento (BLAUSTEIN, 1977).

A principal fonte de $Na^+$ do organismo é o consumo de cloreto de sódio (NaCl), sal de cozinha ou de mesa (40% de $Na^+$), que habitualmente são adicionados aos alimentos de origem protéica, grãos e vegetais que já contem $Na^+$ naturalmente. O $Na^+$ tem uma boa absorção no trato gastrointestinal e sua eliminação se dá principalmente pelo sistema renal através da urina (90–95%) e em menor parte pelas fezes e pelo suor (MAHAN & ESCOTT-STUMP, 2005).

O processamento dos alimentos descritos em relatos históricos em que o homem necessitava preservar pescados, carnes e derivados do leite, por exemplo, ou nos tempos atuais, realizados pelas indústrias, tem uma característica em comum: o alto teor de sódio. Ao primeiro, adicionava-se uma elevada quantidade de sal e ao segundo, conservantes com elevado teor de sódio que extrapola a quantidade necessária de ingestão diária. Isso tem acarretado prejuízos à saúde humana, traduzidos com o aumento nos índices de morbi-mortalidade associadas aos distúrbios cardiovasculares relacionados à alta ingestão de sódio (FRISOLI *et al*., 2012; HA, 2014).

Atualmente, as mudanças do estilo de vida e dos hábitos alimentares, como a alta ingestão de gordura, carboidratos e sal (JEW *et al*., 2009; KONNER & EATON, 2010), têm

ocasionado alterações fisiológicas no homem moderno e isto tem sido associado com o aumento da incidência de doenças cardiovasculares e renais (FRISOLI *et al*., 2012).

## 2.1 Os impactos da alta ingestão de NaCl nos sistemas cardiovascular e renal

Os sistemas cardiovascular e renal são fortemente impactados pela sobrecarga de sódio. O sódio estimula a sede e como consequência aumenta a ingestão de líquido. O excesso de líquido, se for somado ao volume sanguíneo presente nos vasos pode gerar a um quadro de expansão de volume extracelular. Para que esse quadro não se estabeleça o coração e os rins desencadeiam respostas fisiológicas para promover a excreção deste excesso de sódio e água no organismo. Quando as respostas fisiológicas desencadeadas pelo elevado consumo de sódio não conseguem restabelecer o volume sanguíneo ocorre o aumento da pressão arterial (FRISOLI *et al*., 2012).

O elevado consumo de sódio impacta o coração com hipertrofia ventricular, fibrose intersticial e ativação local do SRAA. A hipertrofia pode ser dependente ou não da hipertensão (LIMA *et al*., 2006; FERREIRA *et al*., 2010). Parece que uma maior densidade e ativação da isoforma 1 do receptor de AngII, o $AT_1R$, está relacionada à hipertrofia ventricular, independentemente da hipertensão arterial (FERREIRA *et al*., 2010).

A sobrecarga de sódio também se correlaciona positivamente com o estresse oxidativo. Em ratos espontaneamente hipertensos (SHR) que receberam uma dieta rica em sódio tiveram a hipertensão exacerbada e esse efeito foi acompanhado de fibrose perivascular, maior produção de radicais superóxido na aorta e artéria renal e menor atividade da superóxido dismutase (DE CAVANAGH *et al*., 2010).

Os rins também são afetados negativamente com a alta ingestão de sódio. Hipertrofia renal, glomeruloesclerose, hipertrofia glomerular, infiltração de macrófagos, deposição de colágeno no espaço túbulo intersticial e proteinúria têm sido relatados em casos de sobrecarga de sódio (LARA *et al*., 2012). Na hemodinâmica renal, ocorre redução do fluxo plasmático renal e ritmo de filtração glomerular também têm sido relatados (ROCCO *et al*., 2008). Ainda no rim, a hipertrofia glomerular diante da sobrecarga de sódio tem sido foi associada à ativação de proteínas quinases que estão envolvidas na proliferação celular (HAMAGUCHI, *et al*., 2000).

O alto teor de $Na^+$ no líquido extracelular ainda provoca inibição da bomba ($Na^+$-$K^+$)ATPase das membranas plasmáticas e esse evento aumenta sua concentração intracelular que por sua vez leva ao influxo do íon $Ca^{2+}$ nas células da musculatura lisa vascular, gerando a contração dessas células e aumento da resistência vascular periférica (ADROGUÉ & MADIAS, 2007), o que pode contribuir para a elevação da pressão arterial.

### 2.1.2 Papel do estresse oxidativo no desenvolvimento de doenças cardiovasculares e renais

O estresse oxidativo é definido como um desequilíbrio entre a produção de espécies reativas de oxigênio e nitrogênio (ERONs) com as defesas antioxidantes endógenas (JONES, 2006). Estes radicais são produzidos naturalmente pelo metabolismo energético normal e em condições patológicas estão em maior quantidade devido às limitações das defesas antioxidantes. Dessa forma, ocorre um desequilíbrio entre as ERONs e as defesas antioxidantes (JONES, 2006). Nesse contexto, os radicais livres podem reagir com moléculas que formam as estruturas celulares como, por exemplo, as membranas plasmáticas, proteínas e DNA e assim ocasionar a oxidação e a perda de função, especialmente quando a produção

destes ERONs está exercebada. Uma das ERONs, o ânion superóxido, reage com o óxido nítrico e diminui a biodisponibilidade desse importante vasorrelaxante da musculatura lisa vascular (CAI & HARRISON, 2000; RUSH & FORD, 2007). O estresse oxidativo está associado com doenças como a hipertensão arterial (WHITE & SIDHU, 1998; RIZZI *et al*., 2014), a aterosclerose (LI *et al*., 2014), o remodelamento cardíaco e o aumento do tônus da microvasculatura renal, o que acarreta modificações da hemodinâmica glomerular (WILCOX, 2005).

O estresse oxidativo pode impactar o desenvolvimento fetal através das alterações na disponibilidade de substâncias vasoativa. Nesse contexto, a estimulação de tromboxano (WALSH *et al*., 1993) e a diminuição na síntese de prostaciclina (WALSH *et al*., 2004) acarretam vasoconstrição, e diminuem o aporte sanguíneo para o feto.

## 2.2 Programação fetal de doenças crônicas e as possibilidades de reversão

A integridade do ambiente intra-uterino é fundamental para o desenvolvimento fetal. Nas últimas décadas tem emergido a relação do desenvolvimento fetal em um ambiente adverso com a programação de doenças crônicas na idade adulta (BARKER *et al*., 1993; ZANDI-NEJAD *et al*., 2006; PISANESCHI *et al*., 2013). Desnutrição materna (VIEIRA-FILHO *et al*., 2014), exposição materna a glicocorticoides (ORTIZ *et al*., 2003) e a sobrecarga de sódio (CONTRERAS *et al*., 2000) têm sido associadas com a programação da hipertensão na prole quando esses eventos ocorrem no período do desenvolvimento.

O aumento da ingestão dietética de sódio materna inibe o SRAA (BEAUSEJOUR *et al*., 2003) e aumenta o estresse oxidativo placentário (BEAUSÉJOUR *et al*., 2007), alterações que podem perturbar o desenvolvimento fetal. Na prole de mães submetidas a uma sobrecarga de sódio foi demonstrado diminuição dos marcadores de desenvolvimento renal (BALBI *et al*.,

2004) e o números de néfrons (KOLEGANOVA *et al*., 2011). A sobrecarga de sódio pré-natal programa aumento do volume plasmático (CARDOSO *et al*., 2009), a elevação da pressão arterial (CONTRERAS *et al*., 2000; SILVA *et al*., 2003; PORTER *et al*., 2007; KOLEGANOVA *et al*., 2011), a glomeruloesclerose (MARIN *et al*., 2008), a proteinúria, a diminuição do ritmo de filtração glomerular, (MARIN *et al*., 2008; CARDOSO *et al*., 2009; KOLEGANOVA *et al.,* 2001) e o estresse oxidativo renal aumentado (CARDOSO *et al*., 2009).

A hipertensão programada durante a vida intra-uterina em ratos pode ser prevenida quando a ingestão dietética de sódio é reduzida (STEWART *et al*., 2009). Manning & Vehaskari (2005) demonstraram que a administração de enalapril, um inibidor de enzima conversora de angiotensina (IECA), previne o desenvolvimento da hipertensão programada pela desnutrição intra-uterina. SHR submetidos ao tratamento durante o período perinatal com L-arginina e tempol, um mimético da superóxido dismutase, apresentam redução nos níveis pressóricos (DE QUEIROZ *et al*., 2010). Stewart *et al*., (2005) demonstraram que ratos quando tratados com tempol, não desenvolvem hipertensão programada durante o desenvolvimento fetal. Nesse estudo, os autores enfatizam o papel do estresse oxidativo e da presença de infiltrado inflamatório no tecido renal, como responsáveis pela hipertensão. Dados do nosso laboratório demonstram que o α-tocoferol, administrado durante o aleitamento, previne alterações da via de sinalização da AngII que pode estar associada a hipertensão programada pela desnutrição materna durante a gravidez (VIEIRA-FILHO *et al*., 2011; VIEIRA-FILHO *et al*., 2014).

A administração de antioxidante durante o período de desenvolvimento emerge uma possibilidade de reversão de doenças programadas por condições maternas adversas. Nesse contexto o α-tocoferol parece ser um candidato promissor.

## 2.3 Desenvolvimento renal

A nefrogênese é um evento em que fatores de crescimento que atuam de forma específica desencadeiam a interação, a diferenciação e a maturação das células que formarão o tecido renal. Em ratos, a nefrogênese tem início no 12º dia de vida embrionária e conclui-se entre o 13º e o 15º dia de vida pós-natal. Em humanos, inicia-se a partir da 5ª semana e se conclui antes do término da 36ª semana de gestação (REEVES *et al.*, 1978; NIGAM *et al*., 1996). O rim primitivo sofre transformações durante a organogênese e são denominados pronefro, mesonefro e metanefro (NIGAM *et al*., 1996).

O pronefro é uma estrutura rudimentar e não apresenta função. Os ductos do pronefro servirão de suporte para o desenvolvimento do mesonefro. Este por sua vez exerce a função de órgão excretor por um curto período de tempo e regride com a evolução do desenvolvimento embrionário. O metanefro constituirá o rim definitivo. Este é formado por células mesenquimais indiferenciadas, e ao interagir com as células epiteliais do broto uretérico são induzidas a se diferenciar em células epiteliais especializadas que originarão o epitélio tubular do nefro. Esta etapa do desenvolvimento é chamada de transição mesênquima-eptelial (BARD, 2003). Células mesequimais do metanefro liberam o fator neurotrófico derivado da glia, o GDNF, que exerce sua ação através da ativação do receptor de membrana tirosina quinase, c-Ret. Essa interação mútua é importante na ramificação do broto uretérico (YOSYPIV 2008; SONG *et al*., 2010).

### 2.3.1 Inibidor da enzima conversora de angiotensina e o desenvolvimento renal

Todos os componentes do SRAA são expressos no tecido renal fetal e adulto: angiotensinogênio, enzima conversora de angiotensina, AngII e os receptores de AngII $AT_1$ e $AT_2$ (BURNS *et al*., 1993). Estudos realizados em animais geneticamente modificados que não expressam angiotensinogênio, enzima conversora de angiotensina (ESTHER *et al*., 1996; KAKINUMA *et al*., 1999) ou através do bloqueio farmacológico do SRAA tem reforçado a importância da integridade do SRAA no desenvolvimento dos rins (FRIBERG, *et al*., 1994; LASAITIENE *et al*., 2004; MACHADO *et al*., 2008; FANELLI *et al*., 2011; MARIN *et al*., 2011). As alterações renais observadas em ratos provenientes de mães que receberam antagonista de receptor $AT_1$, o losartan, durante a lactação são redução no número de nefros, aumento da pressão hidrostática glomerular, esclerose glomerular, inflamação, expansão intersticial, apoptose, albuminúria e prejuízos nos mecanismos de concentração urinária (FRIBERG, *et al*., 1994; GOMES *et al*., 2008; FANELLI *et al*., 2011; MARIN *et al*., 2011). Lasaitiene *et al*., (2004) estudaram ratos que receberam por via intraperitoneal losartan nos primeiros dez dias de vida pós natal e demonstraram alterações funcionais no ramo espesso da alça ascendente de Henle que, em parte, justificaria os distúrbios nos mecanismos de concentração e diluição urinária observados nos estudos acima citados. Nesse mesmo estudo foi demonstrado um aumento na expressão da COX-2, o que pode ser correlacionado à resposta inflamatória que foi observada na prole de mães que receberam losartan durante a lactação. Em outro trabalho, Lasaitiene *et al*., (2006) demonstraram que ratos tratados do 2º ao 9º dia de vida pós natal com enalapril apresentam rompimento da membrana interna da mitocôndria e redução de um componente estrutural da cadeia transportadora de elétrons, a citocromo C. Esse resultado sugere que as alterações observadas ao nível mitocondrial

comprometem a produção de energia pelas células em desenvolvimento e esse evento comprometeria a formação dos nefros.

Evidências clínicas da utilização de losartan durante a gestação sobre o desenvolvimento renal fetal corroboram com as evidências experimentais. Necropsia de rins fetais, de mães que faziam terapia com losartan cronicamente, mostra aspecto macroscópico normal, porém, com tamanho maior quando comparado aos rins de fetos saudáveis. As observações microscópicas revelaram má formação vascular renal com pouco desenvolvimento da *vasa recta* e espessamento da parede dos vasos arteriolares. Os glomérulos apresentam-se isquêmicos, retraídos e de aspecto floculoso. Os segmentos epiteliais que formam os túbulos, proximal e distal, também têm seu desenvolvimento comprometido. (MARTINOVIC *et al*., 2001; DAÏKHA-DAHMANE *et al*., 2006).

A AngII exerce seu efeito como regulador do desenvolvimento renal através da ativação da proteína quinase ativada por mitógenos (MAPK) (KUBO *et al*., 2001). A MAPK faz parte de uma família de proteínas-quinases representada pela proteína quinase ativada por sinal extracelular (ERK), quinase N-terminal c-jun (JNK) e a MAPK p38. Em suma, a ERK promove proliferação e diferenciação celular (OMORI *et al*., 2000; IHERMANN-HELLA *et al*., 2014) enquanto a JNK e MAPK p38 induzem apoptose (CHOI *et al*., 2005). Assim, a interação da AngII com seus receptores juntamente com ativação de seus mensageiros intracelulares modulam o desenvolvimento renal (BALBI *et al*., 2009).

No entanto, IECA quando administrados por um curto período de tempo após o término da lactação tem efeitos benéficos. Manning & Vehaskari, (2005) demonstraram que o tratamento com IECA por um período curto de três semanas previne a hipertensão previamente programada pela restrição dietética materna durante a gestação.

## 2.4 Função renal e as adaptações moleculares envolvidas na reabsorção de $Na^+$

No túbulo proximal são reabsorvidos cerca de 65-70% do sódio filtrado, a maior parte através de transporte ativo secundário na membrana apical, graças ao gradiente eletroquímico gerado pela bomba de sódio ($Na^+$+$K^+$)ATPase presente na membrana basolateral. A ($Na^+$+$K^+$)ATPase é um heterodímero formado pelas subunidades α, β e γ. A subunidade α apresenta um sítio de ligação sensível à ouabaína e apresenta 4 isoformas ($\alpha_1$, $\alpha_2$, $\alpha_3$ e $\alpha_4$) expressas em vários tecidos. A forma predominante nas células epiteliais renais é a isoforma $\alpha_1$ (SUMMA *et al*., 2004). A subunidade β apresenta uma parte de sua estrutura altamente glicosilada e é responsável pela integração da ($Na^+$+$K^+$)ATPase com a membrana plasmática, bem como pela atividade enzimática deste transportador (TAUB *et al*., 2010).

O teor de sódio na dieta e a AngII modulam a atividade da ($Na^+$+$K^+$)ATPase no túbulo proximal e em outros segmentos do túbulo, como ramo ascendente da alça de Henle e túbulo distal. Os rins em condições normais mantêm o balanço de sódio e água durante uma sobrecarga de sódio vigente, através de diurese e natriurese aumentados, devido à redução de transportadores de sódio na membrana apical com subseqüente redução da ($Na^+$+$K^+$)ATPase na membrana basolateral (SONG *et al*., 2004; YANG *et al*., 2008). Diante de sobrecarga de sódio, o trocador $Na^+$-$H^+$ (NHE3) no túbulo proximal sofre fosforilação e migra para a base dos microvilos, enquanto no ramo ascendente da alça de Henle e no túbulo distal ocorre retração do co-transporte $Na^+K^+2Cl^-$ e de canais de sódio (ENaC), respectivamente, para as vesículas intracelulares (PERIYASAMY *et al*., 2005; YANG *et al*., 2008). Diante dos ajustes que ocorrem na membrana apical, a densidade da ($Na^+$+$K^+$)ATPase torna-se diminuída na membrana basolateral do epitélio tubular (YANG *et al*., 2008). Adicionalmente, ocorre

internalização de receptores $AT_2$ para o meio intracelular. Todas estas alterações contribuem para a diminuição da reabsorção tubular de sódio (YANG *et al*., 2008).

A AngII é um hormônio que atua no túbulo proximal através de dois receptores, o $AT_1$ e o $AT_2$ (GILDEA, 2009). Este hormônio apresenta efeitos bifásicos sobre a atividade da $(Na^+ + K^+)$ATPase; em baixas concentrações ela aumenta a atividade dessa bomba no túbulo proximal (GARVIN 1991; YINGST *et al*., 2004), enquanto em altas concentrações tem efeito oposto (HARRIS *et al*., 1977). Assim, em concentrações fisiológicas, a AngII aumenta a reabsorção tubular proximal de sódio. Quando a pressão de perfusão renal está diminuída, a AngII aumenta a densidade da $(Na^+ + K^+)$ATPase na membrana basolateral (YINGST *et al*., 2009). Há evidências de que este hormônio aumenta a atividade da $(Na^+ + K^+)$ATPase também de forma indireta, atuando primeiramente em transportadores da membrana apical, como o NHE3, levando ao aumento da concentração de $Na^+$ intracelular e subsequente aumento da atividade da $(Na^+ + K^+)$ATPase (REILLY *et al*., 1995; WONG, *et al*., 1996). A AngII tem ainda um efeito direto sobre a $(Na^+ + K^+)$ATPase, através da diminuição da sensibilidade à ouabaína bem como através de alterações no seu estado de conformação e fosforilação (YINGST *et al*., 2004).

Quando os mecanismos moleculares e humorais não conseguem manter o balanço entre natriurese e antinatriurese, a ingestão elevada de sódio pode induzir hipertensão (JAITOVICH *et al*., 2010). SHR desenvolvem hipertensão entre a 4ª e a 6ª semanas de vida. É possível que a hipertensão desenvolvida por esta linhagem de ratos seja, em parte, devido à atividade aumentada da $(Na^+ + K^+)$ATPase (GARG *et al*., 1985) no túbulo proximal. Alem disso tem sido observado que a capacidade de inibição desta bomba pela dopamina em SHR está diminuída em comparação aos ratos Wistar Kyoto. Há também alterações na expressão e distribuição das subunidades da $(Na^+ + K^+)$ATPase como $\alpha_1$ e $\gamma$ (HINOJOS *et al*., 2004). O aumento da expressão protéica da subunidade $\gamma$ aumenta a afinidade da $(Na^+ + K^+)$ATPase a

molécula de ATP (ARYSTARKHOVA *et al*., 1999; THERIEN *et al*., 1999) e leva ao aumento da reabsorção de sódio no túbulo proximal em SHR (MAGYAR *et al*., 2000). Além das alterações observadas na ($Na^+$+$K^+$)ATPase, outros estudos têm demonstrado alterações na atividade do trocador $Na^+$-$H^+$ no túbulo proximal destes animais (GARG *et al*., 1985; BEACH *et al*., 1990; DAGHER *et al*., 1992; LAPOINTE *et al*., 2002).

Outra enzima (bomba) presente na membrana basolateral do túbulo proximal é a $Na^+$-ATPase, a qual também participa da homeostase de sódio e apresenta as peculiaridades de ser insensível à ouabaína e sensível à furosemida. A $Na^+$-ATPase é responsável pelo ajuste fino da reabsorção de sódio no túbulo proximal e sua atividade é regulada pela AngII e seus metabólitos biologicamente ativos como a angiotensina 3-4 e a angiotensina 1-7 (CARUSO-NEVES *et al*., 2001; RANGEL *et al* 1999, 2002, 2005). De forma semelhante ao que ocorre com a ($Na^+$+$K^+$)ATPase, baixas concentrações de AngII aumentam a atividade da $Na^+$-ATPase, enquanto concentrações elevadas têm efeito oposto. Foi demonstrado em SHR que a atividade da $Na^+$-ATPase está aumentada na 14ª semana de vida, momento a qual a hipertensão está estabelecida (QUEIROZ-MADEIRA *et al*., 2009). No entanto, nessa mesma linhagem foi demonstrado que a AngII diminui a atividade da $Na^+$-ATPase e que esta inibição pode estar relacionada com a ativação de receptores AT2 (QUEIROZ-MADEIRA *et al*., 2009).

**2.5 Desenvolvimento cardíaco**

Em condições normais, o coração é o primeiro órgão interno a ser desenvolvido durante a vida embrionária devido à sua importância funcional em bombear sangue para manter os níveis de oxigenação e nutrição adequados aos demais tecidos, uma vez que nessa etapa do desenvolvimento há uma alta taxa de proliferação celular (DRENCKHANH, 2009) e

o processo de difusão simples dos metabólitos gerados pelos tecidos em formação não é suficiente para manter a homeostase. Os cardiomiócitos (CMs) são células altamente especializadas que formam o tecido cardíaco: células de trabalho, gênese e condução da atividade elétrica do coração. Os CMs apresentam peculiaridades que são observadas durante as etapas embrionária, fetal e pós-natal que podem ser divididas em hiperplasia, binucleação e hipertrofia (LI *et al*., 1996). Estas células se desenvolvem inicialmente na estrutura primitiva cardíaca – tubo cardíaco. Durante a vida intra-uterina e nos primeiros dias de vida pós natal (LI *et al*., 1996) os CMs apresentam uma alta taxa de proliferação celular e síntese de ácido desoxirribonucléico (DNA) (citocinese). Após o nascimento, cerca de 3 a 4 dias de vida pós-natal, ocorre cariocinese e, diferente do que é observado no ambiente intrauterino, não ocorre a citocinese, o que resulta em CMs binucleados (LI *et al*., 1996; SOONPAA *et al*., 1996; LI *et al*., 1997). Estes por sua vez serão a população de células que constituirão o tecido cardíaco adulto e a porcentagem representativa varia entre as espécies. Em humanos, as células binucleadas representam 25–50% (Schmid *et al*., 1985; Olivetti *et al*., 1996) e em ratos cerca de 90% dos CMs são binucleados (SOONPAA *et al*., 1996; CLUBB *et al.*, 1984).

Os CMs respondem a fatores de crescimento que ativam mensageiros intracelulares específicos e resultam em proliferação celular como, por exemplo, a AngII (SUNDGREN *et al*., 2003; MEL'NIKOVA *et al*., 2006), o cortisol (GIRAUD *et al*., 2006) e a isoforma 1 do fator de crescimento semelhante à insulina (IGF-1) (Kajstura *et al*., 1994). Por outro lado, o peptídeo atrial natriurético (PAN) e tri-iodo-L-Tironina ($T_3$) suprimem a atividade mitótica dos CMs (SUNDGREN *et al*., 2003).

O desenvolvimento cardíaco no ambiente intra-uterino ocorre em baixa $pO_2$ que estimula a expressão de uma família de fatores de transcrição gênica, o fator induzível por hipóxia (HIF-I). O HIF-1 é responsável por desencadear adaptações celulares à redução da $pO_2$ como, a regulação da expressão gênica do fator de crescimento derivado do endotélio

(VEGF), responsável pela angiogênese e remodelamento cardíaco fetal (PATTERSON & ZHANG, 2010), bem como enzimas envolvidas na via glicolítica (LOPASCHUK & JASWAL, 2010) que são importantes para manutenção da produção de trifosfato de adenosina (ATP).

O sistema adrenérgico é de suma importância para o desenvolvimento do coração durante o desenvolvimento fetal, bem como nas etapas iniciais do desenvolvimento pós-natal. A infusão contínua de propanolol (antagonista β adrenérgico não seletivo) em ratas, nas duas últimas semanas de gestação altera o desenvolvimento cardíaco com redução da síntese de DNA (KUDLACZ, *et al*., 1990). Tseng *et al.*, (2001) demonstraram que em ratos recém nascidos, a administração de propranolol após 3 dias do nascimento diminuiu a proliferação dos CMs. Fato que foi associado à diminuição na via de sinalização intracelular a p70 S6 quinase. Em paralelo, Wadhawan *et al*., (2003) demonstraram que em coração fetal de ratos a promotora da expressão do receptor β-1 adrenérgico está integrada à ativação de genes que estão relacionados com o desenvolvimento celular cardíaco e sua adaptação pós-natal.

### 2.5.1 Sobrecarga de sódio pré-natal e desenvolvimento cardíaco fetal

Existem evidências que apontam uma correlação negativa entre o consumo elevado de sódio durante o período pré-natal e o desenvolvimento cardíaco (DING *et al*., 2010; PIECHA *et al.*, 2012; ALVES-RODRIGUES *et al*., 2013). Alterações ultra-estruturais no coração fetal, como desorganização das fibras contráteis, redução no tamanho das mitocôndrias, perda das cristas mitocondriais, presença de vacúolos e hipertrofia cardíaca foram observados na prole de mães que foram submetidas a uma dieta com elevado teor de sódio (DING *et al*., 2010). Em paralelo com as alterações morfológicas, esse mesmo estudo mostrou aumento na

expressão de RNAm e na expressão da proteína para o receptor de AngII, o $AT_1$ (DING *et al.*, 2010). Interessante é que essas alterações foram correlacionadas com mudanças epigenéticas para o mesmo receptor. Nesse mesmo estudo foi demonstrado ainda uma maior quantidade de AngII no tecido cardíaco e proliferação celular (maior número de células na fase S do ciclo celular). Outro estudo demonstrou que ratos submetidos à sobrecarga de sódio pré natal apresentam na idade juvenil hipertrofia e aumento da freqüência cardíaca (PIECHA *et al.*, 2012). Diferente do que foi observado no estudo de Ding *et al*., (2010), as alterações da expressão dos componentes do SRAA não foram observadas na idade adulta (ALVES-RODRIGUES *et al*., 2013). Portanto, é provável que haja uma janela de desenvolvimento e maturação do tecido cardíaco após o término do período pré-natal.

## 2.6 Homeostase do $Ca^{+2}$ intracelular na modulação da contração cardíaca

A função primordial do coração é gerar um gradiente de pressão favorável a hemodinâmica do volume sanguíneo a fim de manter a perfusão tecidual adequada e promover a homeostase dos tecidos periféricos (THORNBURG *et al*., 2010). Para este fim, as proteínas contráteis presentes nos CMs interagem e se movimentam de forma organizada, gerando a contração muscular (WOODCOCK & MATKOVICH, 2005). Todos esses eventos ocorrem de forma organizada através da interação do potencial de ação cardíaco com o influxo de $Ca^{+2}$. O balanço desse íon é muito importante para a contração muscular adequada. Perturbações nos mecanismos intracelulares que regulam a sua concentração levam a ocorrência de comprometimento no mecanismo de acoplamento excitação-contração (BÖGEHOLZ *et al*., 2012).

A contração do CMs é dependente da geração do potencial de ação que se inicia pela entrada de $Na^{+}$ na célula e precede o desenvolvimento da força de contração. O acoplamento

excitação-contração é dependente da elevação dos níveis de $[Ca^{+2}]i$. Na excitação, canais de $Ca^{+2}$ do tipo L regulados por voltagem se abrem, permitindo a entrada desse íon na célula. Esse evento induz a liberação de $Ca^{+2}$ pelo retículo sarcoplasmático através dos receptores de rianodina, elevando a $[Ca^{+2}]_i$ em cerca de 100 vezes. Finalmente, a elevação da $[Ca^{+2}]i$ induz a contração da célula e ocorre a sístole. Por outro lado, a diástole depende da remoção do $Ca^{+2}$ citosólico para o meio intersticial e, principalmente, para o retículo sarcoplasmático. O primeiro processo é dependente, sobretudo, do trocador $Na^{+}/Ca^{+2}$ presente no sarcolema, contudo a $Ca^{+2}$-ATPase de membrana plasmática (PMCA) também auxilia esse processo. A recuperação do $Ca^{+2}$ para o retículo sarcoplasmático também é realizada por uma $Ca^{+2}$-ATPase, a SERCA (sarco/endoplasmic reticulum $Ca^{+2}$-ATPase). Assim como a contratilidade, a velocidade e a força de contração ocorrem em função da $[Ca^{+2}]i$. Dessa forma, a cada batimento o efluxo do $Ca^{+2}$ citosólico deve ser igual ao influxo do íon (EISNER *et al*., 2013).

A corrente de $Ca^{+2}$ é determinante para o acoplamento excitação-contração. Isto requer que os principais componentes que modulam esse evento, o receptor de rianodina e a SERCA, funcionem de forma coordenada para manter as concentrações de $Ca^{+2}$ compatíveis com os estágios de contração e relaxamento sincronizados. Quando a homeostase do $Ca^{+2}$ intracelular é comprometida disfunções na contratilidade miocárdica são deflagradas. Patologias como diabetes, hipertensão, insuficiência cardíaca e arritmias são associadas com alterações na contração cardíaca. Na diabetes, a disfunção cardíaca está associada à diminuição da força de contração que, que por sua vez está associada à menor atividade da SERCA e sua expressão protéica (BELKE & DILLMANN, 2004; BAI *et al.*, 2012).

Dupont *et al.*, (2012) estudaram SHR em dois momentos diferentes: a fase pré hipertensiva e após o estabelecimento da hipertensão. Eles demonstraram que o

comprometimento da função diastólica desses animais estava correlacionado à menor expressão da SERCA, com redução da sua atividade.

A insuficiência cardíaca está associada com alterações da homeostase do $Ca^{+2}$ intracelular. Menor atividade da SERCA e sua expressão protéica foram observadas (BELEVYCH *et al*., 2007; ZIMA *et al*., 2014).

O estresse oxidativo, quando estabelecido, modula negativamente a atividade da SERCA. Este quadro é comumente observado em doenças como, diabetes, hipertensão e insuficiência cardíaca, o que sugere que o estresse oxidativo pode ser uma via aditiva na disfunção da atividade da SERCA e que pode contribuir para as alterações dos mecanismos de excitação-contração cardíaca presentes nestas doenças (SAG *et al*., 2013).

## 2.7 α-tocoferol: estrutura química, mecanismos de ação e importância fisiológica

O α-tocoferol é um dos 8 análogos que são denominados de vitamina E. São encontrados em plantas e de forma geral podem ser divididos em duas grandes famílias: tocoferois e tocotrienois, ambos representados pelos homônimos (α, β, γ e δ). As propriedades e ações biológicas diferem entre os tocoferois, sendo o α-tocoferol o mais ativo biologicamente (KAISER *et al*., 1990). Isto pode ser correlacionado a sua conjugação preferencial às lipoproteínas sintetizadas no tecido hepático e sua posteriormente liberação para corrente sanguínea (TRABER & KAYDEN, 1989). A estrutura química dos tocoferois e tocotrienois estão apresentadas na figura 1. Os tocoferois apresentam uma cadeia fitil com 3 carbonos quirais e os tocotrienois uma cadeia insaturada. Outra diferença estrutural são os 2 radicais do anel aromático que estão apresentados no box da Figura 1.

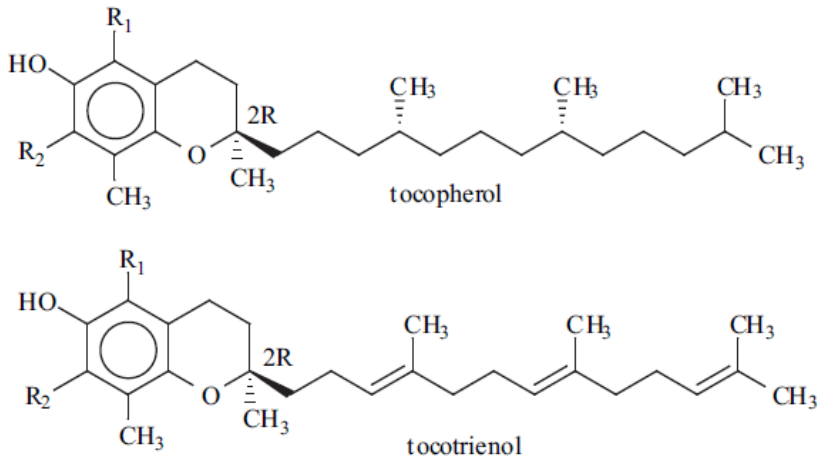
De uma forma geral, a principal fonte da vitamina E são os óleos vegetais e esta fonte representa mais da metade do tocoferol ingerido através da dieta. Em particular o α-tocoferol

é mais abundante no azeite de oliva e no óleo de girassol (ZINGG & AZZI, 2004). Durante a lactação, é transferido através do leite materno para o lactente (DEBIER & LARONDELLE, 2005). Os níveis plasmáticos dos tocoferois são determinados pela sua absorção na parte inicial do intestino, de onde são distribuídos para os tecidos periféricos e fígado (ZINGG & AZZI, 2004). O fígado expressa a proteína de transferência do α-tocoferol, a α-TPP, que é imprescindível para circulação intracelular do tocoferol, retenção nos hepatócitos e incorporação à lipoproteínas de baixa densidade (VLDL). A distribuição dos tocoferois a partir do tecido hepático é realizada quando ocorre secreção hepática de VLDL. Desta forma ocorre distribuição através da corrente sanguínea para os tecidos periféricos (ZINGG & AZZI, 2004). A distribuição do α-tocoferol nos tecidos é variável: uma maior quantidade é encontrada no tecido adiposo e glândula adrenal e uma menor quantidade são encontradas nos rins e coração (BAUERNFEIND *et al*., 1970; TRABER *et al*., 1984).

A ação molecular clássica do α-tocoferol é sua função como anti-oxidante, através da sua reação com radicais superóxidos ($O_2^{\bullet -}$) originados do metabolismo celular normal, produzidos por exemplo, pela enzima pró-oxidativa NADPH-oxidase (AZZI, 2007). Estes radicais podem reagir com outras moléculas presentes nas estruturas celulares, como lipídeos e proteínas, o que pode resultar em dano celular. O α-tocoferol reage com moléculas oxidadas e previne que reações deletérias subseqüentes venham a ocorrer. Por fim, o α-tocoferol é reciclado através de sua redução por enzimas anti-oxidantes endógenas, como por exemplo a glutationa (WASHBURN & WELLS, 1999), ou ainda pelo ácido ascórbico (VILLACORTA *et al*., 2007)

Por outro lado, algumas ações moleculares do α-tocoferol não estão associadas à sua ação clássica. Tem-se elucidado suas ações celulares independentes da sua ação anti-oxidante através da modulação e interação com enzimas de sinalização intracelular, proteínas estruturais e fatores de transcrição gênica. O α-tocoferol diminui a proliferação de células

musculares lisas (BOSCOBOINIK *et al*., 1995) e a atividade da proteína quinase C (PKC) (AZZI *et al*., 1998), aumenta a expressão e a atividade da fosfolipase $A_2$ (TRANK *et al*., 1997; WU *et al*., 2005), diminui a expressão da ciclo-oxigenase 2 (COX-2) (Vieira-Filho *et al*., 2014) e modula a atividade da NADPH oxidase (CHEN *et al*., 2001).




| $R_1$ | $R_2$ | |
|---|---|---|
| $CH_3$ | $CH_3$ | α |
| $CH_3$ | H | β |
| H | $CH_3$ | γ |
| H | H | δ |

Figura 1. Estrutura química e os radicais da molécula do tocoferol e tocotrienol. Fonte: Zingg e Azzi, Non-antioxidante activities of vitamine E. Current Medicinal Chemistry, 2004, 11, 1113–1133.

## 2.8 Enalapril: visão geral

O enalapril é um fármaco da classe dos anti-hipertensivos denominados inibidores da enzima conversora de angiotensina. É um pró-fármaco administrado na forma de maleato de enalapril que após esterificação no fígado, é convertido em seu metabólito ativo, o enalaprilato. A sua ação farmacológica é caracterizada pela inibição da conversão da angiotensina I em angiotensina II pela enzima conversora de angiotensina (RAIA *et al*., 1990). Mediante inibição da produção de AngII ocorre diminuição da resistência vascular

periférica e, consequentemente redução nos níveis pressão arterial (RAIA *et al*., 1990). Além da sua utilização como anti-hipertensivo o enalapril também é utilizado na insuficiência cardíaca, no infarto do miocárdio e na nefropatia diabética (SONG & WHITE, 2002).

Os efeitos benéficos do enalapril vão além de sua ação anti-hipertensiva clássica. SHR que foram tratados durante o período pré-natal com enalapril tiveram a hipertensão atenuada (SOUZA-BONFIM *et al*., 2005). Manning & Vehaskari (2005) demonstraram que ratos submetidos à desnutrição pré-natal quando são tratados com enalapril por 3 semanas após o desmame a hipertensão não é estabelecida.

A alta ingestão de sódio materna durante o período perinatal pode programar na prole distúrbios cardiovasculares e renais. As alterações moleculares envolvidas nesse processo podem ser reprogramadas quando intervenções anti-oxidantes são realizadas no período pós-natal. A Vitamina E consiste em uma das intervenções antioxidantes utilizadas na clínica médica na prevenção da pré-eclampsia e de sua repercussão no concepto. No entanto, os efeitos desse tratamento no desenvolvimento do coração e do rim são pouco conhecidos. Por outro lado evidências experimentais indicam que inibidores da enzima conversora de angiotensina em períodos precoce do desenvolvimento podem reprogramar as alterações renais produzidas pela sobrecarga de sódio materna.

## 3. OBJETIVOS

Avaliar os efeitos da sobrecarga de sódio, da administração de um inibidor da enzima conversa de angiotensina, o elanapril, ou do α-tocoferol durante o período perinatal.

**Objetivos específicos**:

- Investigar os efeitos da sobrecarga de sódio perinatal e a administração do enalapril por três semanas após o desmame, sobre: i) a reabsorção de sódio no túbulo proximal através da avaliação da $(Na^+ + K^+)$ATPase e $Na^+$-ATPase; ii) a ação da angiotensina II sobre a atividade da $Na^+$-ATPase; iii) a via de sinalização da PKC e da PKA e a expressão dos receptores $AT_1$ e $AT_2$ da angiotensina II; iv) a inflamação e a deposição de colágeno do tecido renal.

- Investigar, em ratos controles ou submetidos à sobrecarga de sódio durante o período pré-natal, os efeitos do tratamento com α-tocoferol durante os períodos da lactação ou por três semanas após o desmame sobre: i) a pressão arterial média e frequência cardíaca; ii) a expressão do receptor β-1 e atividade das enzimas $Ca^{+2}$-ATPase PMCA e SERCA no coração; iii) a produção de radicais superóxidos no coração e rim; iv) os parâmetros funcionais renais.

- Avaliar efeitos da sobrecarga de sódio, desde a vida intrauterina até o desmame, associada ao tratamento com enalapril, por três tres semanas após o desmame sobre: i) a pressão arterial média e a frequencia cardíaca; ii) os parâmetros funcionais renais e; iii) o estresse oxidativo renal e hepático.

## CONCLUSÕES

1) A sobrecarga de sódio durante o período perinatal induziu hipertrofia renal e cardíaca, aumento da expressão e atividade da $(Na^{+}+K^{+})$ATPase no túbulo proximal, hiporesponsividade da angiotensina II sobre atividade da $Na^{+}$-ATPase no túbulo proximal, alterações na via de sinalização da angiotensina II no túbulo proximal e inflamação e fibrose no tecido renal. A sobrecarga de sódio durante o período perinatal também levou a hiporesponsividade à ação hipertensiva da angiotensina II.

2) O tratamento com enalapril reverteu várias alterações moleculares produzidas pela sobrecarga de sódio, no entanto não preveniu o processo inflamatório produzido pela sobrecarga de sódio. O tratamento com enalapril também recuperou a ação hipertensiva da angiotensina II, no entanto produziu hipertrofia cardíaca e elevação do estresse oxidativo em ratos controles.

3) O tratamento com α-tocoferol durante a lactação programou hipertensão, atrofia cardíaca e hiporesponsividade à ação hipertensiva da angiotensina II, tanto em ratos controles, como em ratos submetidos ao tratamento com salina durante a vida pré-natal. Estas alterações cardíacas foram associadas com menor expressão do receptor β-1 e alterações na homeostase de $Ca^{+2}$. Quando administrado após o desmame, por três semanas, o α-tocoferol diminuiu a sensibilidade dos baroreflexos em ratos que foram submetidos a sobrecarga de sódio durante o período pré-natal. O tratamento com α-tocoferol durante a lactação não programou alterações no estresse oxidativo cardíaco ou renal. No entanto, o tratamento no período pós-desmame programou a elevação do estresse oxidativo nos ratos que receberam salina no período pré-natal.

Em conjunto estes dados demonstram que tanto o inibidor da enzima conversora de angiotensina quanto o α-tocoferol podem reverter alterações produzidas pela sobrecarga

materna de sódio. Porém estas intervenções não são inertes em animais controles. Ademais, estes dados também demonstram que a janela de programação da função cardiovascular e renal vai além do período de amamentação.

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# ANEXO I

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# Perinatal $Na^+$ Overload Programs Raised Renal Proximal $Na^+$ Transport and Enalapril-Sensitive Alterations of Ang II Signaling Pathways during Adulthood

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## Abstract

***Background:*** High $Na^+$ intake is a reality in nowadays and is frequently accompanied by renal and cardiovascular alterations. In this study, renal mechanisms underlying perinatal $Na^+$ overload-programmed alterations in $Na^+$ transporters and the renin/angiotensin system (RAS) were investigated, together with effects of short-term treatment with enalapril in terms of reprogramming molecular alterations in kidney.

***Methodology/Principal Findings:*** Male adult Wistar rats were obtained from dams maintained throughout pregnancy and lactation on a standard diet and drinking water (control) or 0.17 M NaCl (saline group). Enalapril (100 mg/l), an angiotensin converting enzyme inhibitor, was administered for three weeks after weaning. Ninety day old offspring from dams that drank saline presented with proximal tubules exhibiting increased $(Na^+ + K^+)$ATPase expression and activity. Ouabain-insensitive $Na^+$-ATPase activity remained unchanged but its response to angiotensin II (Ang II) was lost. PKC, PKA, renal thiobarbituric acid reactive substances (TBARS), macrophage infiltration and collagen deposition markedly increased, and $AT_2$ receptor expression decreased while $AT_1$ expression was unaltered. Early treatment with enalapril reduced expression and activity of $(Na^+ + K^+)$ATPase, partially recovered the response of $Na^+$-ATPase to Ang II, and reduced PKC and PKA activities independently of whether offspring were exposed to high perinatal $Na^+$ or not. In addition, treatment with enalapril *per se* reduced $AT_2$ receptor expression, and increased TBARS, macrophage infiltration and collagen deposition. The perinatally $Na^+$-overloaded offspring presented high numbers of Ang II-positive cortical cells, and significantly lower circulating Ang I, indicating that programming/reprogramming impacted systemic and local RAS.

***Conclusions/Significance:*** Maternal $Na^+$ overload programmed alterations in renal $Na^+$ transporters and in its regulation, as well as severe structural lesions in adult offspring. Enalapril was beneficial predominantly through its influence on $Na^+$ pumping activities in adult offspring. However, side effects including down-regulation of PKA, PKC and $AT_2$ receptors and increased TBARS could impair renal function in later life.

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## Introduction

High $Na^+$ intake is a reality of modern society, particularly due to the use of industrialized products. Rats subjected to maternal $Na^+$ overload during prenatal and lactation periods present with glomerulosclerosis [1], increased proteinuria [2] and hypertension [3,4] as adults. When exposed to $Na^+$ overload during the prenatal period, newborn rats present with reduced expression of several markers of fetal kidney development including angiotensin II (Ang II) [5]. When exposed to $Na^+$ overload from conception to weaning, the renin activity of adult offspring is unresponsive to a high salt intake, *i.e.* high $Na^+$ intake does not suppress renin secretion and Ang II expression is increased in kidneys. Therefore, perinatal $Na^+$ overload leads to renin angiotensin system (RAS) over-activity during adulthood [4]. In addition, an overactive RAS appears to be responsible, at least in part, for the aforementioned renal functional alterations produced by perinatal over-exposure to salt. Furthermore, Ang II increases renal oxidative stress [6,7] that may disturb tubule interstitial microenvironment, leading to structural and functional changes in $Na^+$ transporters [8,9].

On the other hand, kidney development in the rat ends at approximately postnatal day 12 [10], and pharmacological inhibition of RAS during this period causes severe alterations in renal structure and function [11,12,13]. In humans, pharmacological inhibition of RAS during the second and third trimesters of pregnancy causes renal anomalies in offspring [14,15,16]. How-

ever, evidence suggests that short-term inhibition of RAS after weaning in rats could reverse prenatal programmed hypertension induced by maternal undernutrition [17]. In addition, it has been demonstrated that early maternal postnatal treatment with α-tocopherol prevents alterations in proximal tubule $Na^+$ transporters of rats that were subjected to prenatal undernutrition [18]. Beneficial effects of inhibiting RAS after weaning demonstrates that the window of opportunity for imprinting molecular changes that affect renal function in adult life lasts beyond the conclusion of nephrogenesis and weaning [17,18]. Therefore, various related early pathological processes can be reprogrammed to achieve normal profiles during adult life.

ATP-dependent $Na^+$ transporters in the proximal tubule cells are modulated by RAS [19,20]. Perinatal $Na^+$ overload leads to RAS overactivity [4] that promotes increased oxidative stress [6] and may affect the activity of the proximal tubule ATP-dependent $Na^+$ transporters. The present study was designed to determine whether a moderate perinatal $Na^+$ overload produce late elevated tissular lipid peroxidation and local macrophage infiltration in the kidneys of young adulthood. The hypothesis was that these alterations could be associated with, or provoke, molecular alterations in: (i) the proximal tubule ($Na^+$+$K^+$)ATPase and $Na^+$-ATPase; (ii) signaling pathways that link renal Ang II receptors, protein kinases C (PKC) and A (PKA), and active $Na^+$ transporters. Furthermore, this study investigated whether inhibition of RAS for three weeks after weaning could reprogram perinatal programmed alterations in $Na^+$ pumps, Ang II receptors ($AT_1$ and $AT_2$) expression, and the activity of PKC and PKA.

## Materials and Methods

### Animal care

Male Wistar rats were used throughout the study. Animal experimental procedures were approved by the Committee for Ethics in Animal Experimentation of the Federal University of Pernambuco, and carried out in accordance with Committee guidelines (protocol n° 23076.055063/2010-03).

### Materials

Enalapril maleate, thiobarbituric acid, furosemide, ouabain, Ang II, phenylmethanesulfonyl fluoride (PMSF), protein A-agarose and trypsin inhibitor (type II-S) were purchased from Sigma-Aldrich (St. Louis, MO). Calphostin C and PKA inhibitor ($PKAi_{5-24}$) were obtained from Calbiochem (La Jolla, CA). Rabbit and goat polyclonal antibodies against Ang II receptors ($AT_1$ and $AT_2$) and the α-subunit of ($Na^+$+$K^+$)ATPase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and that against the cellular marker for activated macrophages ED1 was obtained from AbD Serotec (Raleigh, NC). The polyclonal rabbit anti-Ang II was purchased from Peninsula Laboratories (San Carlos, CA). Streptavidin-horseradish peroxidase was obtained from Dako LSAB System (Dako Corporation, Carpinteria, CA). Horseradish peroxidase-conjugated anti-rabbit antibody and ECL™ Western blotting system were obtained from GE Healthcare. The EIA commercial kit for plasma angiotensin I (Ang I) determinations was obtained from Bachem (Torrance, CA) and $^{32}P_i$ was purchased from the Brazilian Institute of Energy and Nuclear Research (São Paulo, SP, Brazil). [γ-$^{32}$P] ATP was synthesized as in [21]. The commercial creatinine kit was obtained from Labtest (Lagoa Santa, MG, Brazil). All other reagents were of the highest purity available.

### Animal groups

Seventy-day-old female Wistar rats, weighing 200–250 g, were randomly assigned to a maternal control group or saline group. Until weaning, the maternal control group (n = 4) had free access to tap water and the maternal saline group (n = 4) consumed 0.17 M NaCl (1% w/v). Salt loading *via* drinking water rather than food does not reflect the common situation in humans and does not allow a compensatory increase in free water consumption, and this could be considered as a limitation of the experimental model. In addition, it can evoke an augmented antidiuretic hormone (ADH) release and, consequently, in ADH plasma levels. However, we decided to overload mothers by giving salt in drinking water, aiming to program the fetuses without any maternal compensatory mechanism to correct disturbances in the circulating and tissular $Na^+$. Maternal water intake was measured in a previous work with the same model of $Na^+$ overload [2] and varies between the two dam groups. At pregnancy day 1 it was (in ml/24 h per 100 g body weight) 13.8±4.5 (control) and 27.3±2.4 ($Na^+$-overloaded dams) ($P<0.05$); at day 18 it was 17.8±1.5 and 29.9±4.9 ($P<0.05$) in the respective groups.

Relating water ingestion to the amount of chow consumed (containing 0.3% NaCl), the daily maternal salt intake by $Na^+$-overloaded dams averaged 700 mg. The salt intake in control dams averaged 100 mg per day. This ratio in salt intake regime cannot simply be extrapolated to the clinic. In the case of humans, a normal dietary NaCl averages 7%, whereas a content that varies between 10 and 20% (only two fold higher) is considered high as in some Eastern populations [22] and also in Northeastern Brazil [23]. In contrast, NaCl intake in rats is considered normal at 1.1% and elevated above 6% (*i.e.* six fold higher) as described in different models [4].

Mating was carried out at 90 days of age. Only male offspring was used for experimental protocol. The rats (dams and offspring after weaning) were provided with balanced commercial rodent chow (Purina Agribands, Paulínia, SP, Brazil). The control group (C, n = 19) comprised male offspring from mothers that consumed tap water throughout the study. Male offspring from dams that drank saline throughout the prenatal and lactation periods comprised the S group (n = 24). At birth, litters were culled to eight pups (including females that were used in other experiments after weaning) and maintained until weaning. After weaning (21 days after birth), 11 controls and 13 members of the S group were maintained with tap water. Others from each group were maintained on tap water supplemented with enalapril maleate (E; 100 mg/l) for three weeks, and with pure tap water thereafter; these subgroups were denoted by CE (n = 8) and SE (n = 11), respectively. Rats exposed to enalapril were administered an average of 4 mg per day during the three weeks after weaning. All experiments were carried out on animals aged 90 days.

We took into account the possibility of litter effects and, for this reason, pups from each litter are represented in each of the four experimental conditions. The 5–6 male offspring from each litter coming from the two groups of dams (control and $Na^+$ overloaded) were randomly assigned to one of the two conditions to which they were submitted after weaning (without and with enalapril). Thus, we avoided litter effects and balanced the experimental design by using at least one rat from each litter (control mothers) or two rats from the different litters of $Na^+$-overloaded dams.

### Blood pressure, creatinine clearance, proteinuria, urinary volume and urinary $Na^+$ excretion

After acclimation of rats in the room and to the cage constraint for 30 min, systolic blood pressure (SBP) was measured from age of 25 days to 90 days in awake animals using tail-cuff plethys-

mography (IITC Life Science B60-7/16", Life Science Instruments, Woodland Wills, CA). Acclimation of the animals was carried out along three days before pressure determinations. The room was preserved from noise, the ambient temperature was around 22°C and rats were warmed at 36±2°C. Metabolic cages (Tecniplast Gazzada Sarl, Buguggiate, Italy) were used to collect twenty-four h urine samples for measuring proteinuria, creatinine and $Na^+$. Blood samples were obtained from the caudal artery for creatinine measurements. Urinary protein was measured using the Folin phenol method [24]. Urinary $Na^+$ was measured using an electrolyte analyzer (AVL 9180, Roche Diagnostics GmbH, Mannheim, Germany). Serum creatinine was determined using a commercial kit. 48 h after completion of the metabolic studies, animals were decapitated and the kidneys removed to isolate the *cortex cortices* to obtain tubular plasma membranes (see below); the remaining tissue was used to evaluate thiobarbituric acid reactive substances (TBARS) as a measure of lipid peroxidation, constituting an estimate of renal oxidative stress.

## Evaluation of lipid peroxidation

Lipid peroxidation was assessed in kidney tissue by measuring TBARS according to Buege and Aust [25]. One gram tissue fragments were homogenized in 5 ml of 150 mM KCl in an ice bath. Two ml of 0.375% (w/v) thiobarbituric acid diluted with 15% TCA (w/v) were added to each ml of homogenate. The tubes were sealed and heated to 100°C for 15 min and centrifuged in a clinical centrifuge, and the absorbance of the resulting supernatants was evaluated at 535 nm.

## Isolation of proximal tubule cell membranes

Membranes were isolated as previously described [26] from the *cortex corticis*, a renal region where more than 90% of the cell population corresponds to proximal tubule cells [27,28]. Kidneys were maintained in cold 250 mM sucrose, 10 mM HEPES-Tris (pH 7.4), 2 mM EDTA, 0.15 mg/ml trypsin inhibitor and 1 mM PMSF (solution A). Renal cortex was carefully dissected to eliminate contamination with internal regions of the organ and thin transverse sections (0.5 mm) were cut using a Stadie-Riggs microtome. The fragments were homogenized in 4 ml of solution A per gram of tissue in an ice bath. The homogenate was centrifuged at 755× *g* (15 min) to remove unbroken cells, cell debris and nuclei; the resulting supernatant was centrifuged at 8,500× *g* (20 min) to remove mitochondria and at 35,000× *g* (45 min). The final sediment was resuspended in 250 mM sucrose, aliquoted into tubes and stored at −20°C. Protein content was determined using the Folin phenol method [24] with BSA as a standard, using 2.5% (w/v) SDS to solubilize integral membrane proteins. Control for enrichment with basolateral membranes (3−4 fold with respect to the total homogenate) and for minimal residual contamination with other intracellular membranes were as described elsewhere [26]. The final fraction contained apical membranes at a lower yield than the starting homogenate, as revealed using alkaline phosphatase assays [26]. However, ATP-driven $Na^+$ transporters are exclusively located in the basolateral aspect of the cell membrane. Therefore, there was no attempt to fractionate the samples further. The Percoll gradient method used to separate brush border and basolateral membranes from porcine and ovine kidneys [29,30] results in a very low yield of membranes from rat kidneys. Therefore, avoiding this step allowed a reduced number of rats to be utilized during this study, as recommended by the local Committee for Ethics in Animal Experimentation.

## Measurement of ATPase activities

The activity of ouabain-sensitive ($Na^+$+$K^+$)ATPase was measured colorimetrically using unlabeled ATP. During ($Na^+$+$K^+$)ATPase assays, membranes (0.1 mg/ml final concentration) were pre-incubated with or without 2 mM ouabain in 0.1 ml water at 37°C for 20 min. The assays were supplemented with 50 mM Bis-Tris-propane (pH 7.4), 0.2 mM EDTA, 5 mM $MgCl_2$ and 120 mM NaCl (final concentrations in 0.5 ml assays). The reaction was started by adding a mixture of 5 mM ATP and 24 mM KCl (final concentrations), and stopped after 10 min by adding two vol of 0.1 M HCl-activated charcoal. The ($Na^+$+$K^+$)ATPase activity was calculated as the difference between $P_i$ released in the absence and presence of ouabain. Released $P_i$ was spectrophotometrically measured in an ml aliquot of the supernatant obtained after centrifugation of the charcoal suspension at 1,500× *g* for 5 min.

The ouabain-resistant, furosemide-sensitive $Na^+$-ATPase activity was measured using [$\gamma$-$^{32}$P] ATP (~0.03 MBq/µmol) or unlabelled ATP; each method produced identical results ($P>0.05$) and the measurements were grouped for final statistical analysis. The activity was calculated as the difference between $P_i$ or $^{32}P_i$ released in the absence and presence of 2 mM furosemide. The reaction was started by adding 5 mM ATP (or [$\gamma$-$^{32}$P] ATP) to the membranes (0.2 mg/ml) pre-incubated with 2 mM ouabain, as described above, in the presence of 20 mM HEPES-Tris (pH 7.0), 10 mM $MgCl_2$ and 120 mM NaCl. After 10 min the reaction was stopped. Released $^{32}P_i$ was measured using liquid scintillation counting or spectrophotometrically in ($P_i$) a 0.2 ml aliquot of the supernatants obtained.

In one series of experiments, $Na^+$-ATPase activity was evaluated in basal conditions (no Ang II) and, in other three and simultaneous series of tubes, in the presence of different concentrations of Ang II ($10^{-12}$, $10^{-10}$ and $10^{-8}$ M). The impact of PKA inhibition on the activity of the pump was evaluated using the specific inhibitor $PKAi_{5-24}$ (PKAi) during $Na^+$-ATPase assays.

## Protein kinase C (PKC) and cAMP-dependent protein kinase (PKA)

Protein kinase activities were analyzed by measuring the incorporation of the $\gamma$-phosphoryl group of [$\gamma$-$^{32}$P] ATP (specific activity ~1 MBq/nmol) into histone in the absence and presence of the specific PKC or PKA inhibitors, 100 nM calphostin C and 10 nM PKAi, as described previously [29]. The reaction was started by adding ATP (10 µM) to a reaction medium (0.1 ml) containing 20 mM HEPES-Tris (pH 7.0), 4 mM $MgCl_2$, 1.5 mg/ml histone and 0.7 mg/ml tubule membranes protein. After 2 min, the reaction was stopped by adding 0.1 ml 40% TCA and the samples were immediately placed on ice bath. After vigorous stirring, a 0.1 ml aliquot was removed, filtered through a Millipore filter (0.45 µm pore size) and successively washed with ice-cold 20% TCA and 0.1 M phosphate buffer (pH 7.0). Radioactivity was quantified using a liquid scintillation counter. Protein kinase activities were calculated as the difference between total $^{32}P$ incorporated into histone and incorporation in the presence of calphostin (for PKC) or PKAi (for PKA).

## Immunoprecipitation of Ang II receptors ($AT_1$ and $AT_2$ receptors)

Immunoprecipitation was carried out before immunodetection of $AT_1$ and $AT_2$ receptors, as they are expressed at very low levels in proximal tubule membranes [30]. Isolated membranes (1 mg/ml) were initially solubilized in a sucrose solution containing 0.01% CHAPS for 30 min at room temperature. Primary polyclonal antibodies (1:400 dilution) were mixed with protein

A-agarose, gently stirred for 20 min and supplemented with an equal volume of BSA (1 mg/ml) in 0.01% (w/v) CHAPS. This mixture was added to the isolated membrane samples. After constant stirring overnight at 4°C, the samples were centrifuged at 1,000× *g* for 4 min. The supernatant was retained as a control for the immunoprecipitation procedure. The pellet was washed three times with Tris-buffered saline (TBS) and heated to 100°C in a water bath for 4 min with 40 µl SDS-PAGE sample buffer. After final centrifugation at 10,000× *g* for 2 min, the supernatant was subjected to SDS-PAGE and Western blotting.

### SDS-PAGE and Western blotting

The α-subunit of ($Na^+$+$K^+$)ATPase and the Ang II receptors were detected in the membranes and immunoprecipitates, respectively, using specific antibodies. Sample proteins were separated using SDS-PAGE (10%) and transferred to nitrocellulose membranes at 350 mA. Non-specific binding was prevented by incubating the membranes with 5% non-fat milk diluted in TBS (pH 7.6) for 1 h. The membranes were probed with the corresponding primary antibodies (1:500 dilution) for 1 h at room temperature during gentle stirring, washed three times with TBS containing 0.1% Tween 20, exposed to the secondary antibody, washed and visualized using ECL™.

### Immunohistochemical and morphometrical analysis

Transverse slices of kidneys (3 mm) were fixed in Methacarn for 24 h and maintained in 70% ethanol until encapsulated in paraffin. Sections of 6 µm were used for morphometrical and immunohistochemical analysis of cortical and medullary regions. For morphomometrical studies and evaluation of collagen density, sections were stained with periodic acid-Schiff and Sirius red, respectively. For immunohistochemical evaluation of macrophage infiltration, sections were incubated overnight with a monoclonal antibody anti-ED1 (1:100) in a humid chamber at 4°C, and the reaction product was visualized using a secondary antibody labeled with streptavidin-horseradish peroxidase under a light microscope (Eclipse 400, Nikon, Shanghai, China) coupled to a camera (Evolution, Media Cybernetics Inc., Bethesda, MD). For ED1 and collagen evaluation, 20 images from the medulla and cortex of each kidney were acquired using ×400 and ×200 magnification, respectively. The surface density of collagen and ED1 was identified by a researcher and counted using the Image Pro Plus 4.5.1 software (Media Cybernetics, Bethesda, MD). Immunostaining for Ang II in renal cortical cells was carried out as previously described [31] in slices prepared and incubated with the corresponding antibody (1:200). The results in the graphic representations of collagen and ED1 correspond to the ratio between positive areas and the total area under observation. Ang II-positive cells in the tubulointerstitial region were counted in 60 fields from various kidneys (×100 magnification). Measurement of Ang II-positive cells was carried out using 60 glomeruli randomly chosen from kidneys from different rats (×200 magnification). Similarity among slides from the same rat was used as a criterion of unbiased data. Morphometrical studies concerning glomeruli were performed with 30 images being acquired using ×200 magnification and analyzed using AxioVision software (version 4.8.1.0, Carl Zeiss Imaging Solutions).

### Determination of Ang I levels in plasma

Blood samples (4 ml) obtained after animal decapitation were collected in tubes supplemented with concentrated solutions of EDTA (disodium salt), PMSF and trypsin inhibitor, producing final concentrations of 2.7 mM, 1 mM and 0.15 mg/ml, respectively, in a final volume of 5 ml. After centrifugation at 1,600× *g* the supernatants were frozen at −70°C until determinations were carried out. After thawing, samples were submitted to ELISA following the manufacturer's instructions (protocol III) and the final results were expressed as ng per ml of the original plasma sample.

### Statistical analysis

Differences among groups were analyzed using one-way ANOVA test followed by a Student-Newman-Keuls post test, or two-way ANOVA followed by Bonferroni post test, when corresponding. One-way ANOVA followed by Tukey's test analysis was used to compare the responses of $Na^+$-ATPase to Ang II within groups. Kruskal-Wallis test followed by Dunn's multiple comparison test was used to analyze creatinine clearance, proteinuria and TBARS data, after detection of their departure from normality using the Kolmogorov-Smirnov, D'Agostino & Pearson and Shapiro-Wilk normality tests. GraphPad Prism 5 software (Version 5.01, GraphPad Software, Inc. La Jolla, CA) was used for all statistical analyses. The statistical differences were considered significant at $P<0.05$.

## Results

### Body weight at birth and weaning

The birth body weight of offspring was not affected by maternal saline treatment (C: 6.41±0.18 and S: 6.17±0.19 g), as well as the weaning body weight (C: 49±2 and S: 48±3 g).

### Evolution of systolic blood pressure (SBP) with age

SBP from 25−90 day old rats is presented in Fig. 1. Perinatal $Na^+$ overload does not lead to the onset of hypertension in offspring during the period under study. Three-week daily administration of enalapril from weaning provoked a significant decrease in SBP (compare C and S with CE and SE during the period indicated by the horizontal line), demonstrating that the ACE was impacted by the drug. A complete recovery of control values occurred after treatment was ceased. Furthermore, at 90 days of age, SBP of each group increased by approximately 12% after exposure to ammonia (data not shown) to induce olfactory stress [32]. We tested the effect of ammonia on blood pressure to see whether or not the normalized blood pressure in the programmed offspring responds in the same extent as the other groups to the pressoric olfactory stress, as it was the case.

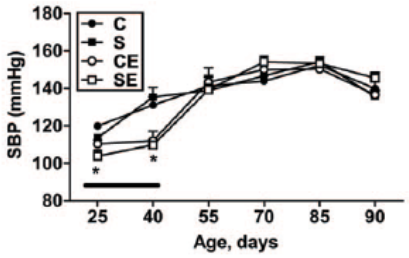


**Figure 1. Systolic blood pressure (SBP) during growth, from age of 25 days to 90 days.** SBP was measured at the ages shown on the *abscissa*. The symbols relating to the groups C, S, CE and SE are indicated in the inset. The continuous horizontal thin black bar indicates the period of daily administration of enalapril to the CE and SE groups. Results are means ± S.E.M. * Statistical difference ($P<0.05$) between CE and SE with respect to C and S.
doi:10.1371/journal.pone.0043791.g001

## Water balance and excretion of $Na^+$ by the offspring

Table 1 depicts results regarding water intake, diuresis and 24 h-urinary $Na^+$ excretion. Alterations in $Na^+$ excretion were observed at 30 days, likely reflecting maternal $Na^+$ overload in the S group and an early and exacerbated response to enalapril in the CE group. Urinary $Na^+$ excretion was similar in all groups at 90 days of age. Water intake and diuresis were similar in all groups and decreased in parallel with age, probably reflecting the elevated water demand at early stages of life.

## $(Na^+ + K^+)$ATPase and $Na^+$-ATPase

Fig. 2A demonstrates that programming caused by perinatal $Na^+$ overload increased expression of the α-subunit of $(Na^+ + K^+)$ATPase in basolateral membranes of proximal tubules from 90 day old offspring (S). Treatment with enalapril for three weeks after weaning resulted in expression of the pump to return to control values (SE), and there was no effect in non-programmed animals (CE). When enzyme activity was measured (Fig. 2B), perinatal $Na^+$ overload programmed elevated activity of $(Na^+ + K^+)$ATPase (S), and treatment with enalapril reprogrammed the enzyme (SE), resulting in activity at levels exhibited by the C group. In contrast, enalapril treatment lowered $(Na^+ + K^+)$ATPase activity by approximately 50% in C animals.

The activity of ouabain-insensitive $Na^+$-ATPase (Fig. 3) was not affected by perinatal $Na^+$ overload (C and S). However, enalapril reduced ouabain-insensitive $Na^+$-ATPase activity in control rats and the progeny of $Na^+$-overloaded mothers (CE and SE) by comparable amounts. $Na^+$-ATPase activity was measured in the presence of different Ang II concentrations (Fig. 4). The effect of the peptide was biphasic in the C group: $10^{-12}$ M Ang II significantly increased $Na^+$-ATPase activity and high concentrations led to progressive inhibition of the previously stimulated activity (upper left panel); without exogenous Ang II, $Na^+$-ATPase activity remained at levels observed in the C group (Fig. 3). The effect of Ang II was lost in the S group at each concentration assayed (upper right panel). In the non-programmed group treated with enalapril (CE, lower left panel), 30%-stimulation using $10^{-12}$ M Ang II was preserved – albeit at lower absolute values – with loss of the biphasic profile. In the SE group (lower right panel), percentual stimulation elicited by $10^{-12}$ M Ang II was maintained at lowered absolute values without significant inhibition at higher concentrations.

**Table 1.** Water balance and excretion of $Na^+$ by the offspring [1].

| | Day | C | S | CE | SE |
|---|---|---|---|---|---|
| Offspring water intake [2] | 30 | 30.5±4.1 | 34.0±1.6 | 29.5±1.5 | 36.3±2.3 |
| | 90 | 10.2±0.4 | 11.4±0.6 | 11.3±0.3 | 11.9±0.5 |
| Offspring water excretion [3] | 30 | 11.6±2.4 | 7.3±0.9 | 10.7±0.9 | 9.3±0.9 |
| | 90 | 3.6±0.2 | 3.5±0.1 | 3.8±0.2 | 3.6±0.2 |
| Offspring urinary $Na^+$ [4] | 30 | 0.47±0.02 | 0.58±0.04 * | 0.72±0.03 *,† | 0.62±0.03 |
| | 90 | 0.57±0.03 | 0.56±0.02 | 0.56±0.02 | 0.54±0.02 |

[1] n = 7–8; 30 and 90 indicate postnatal day of life.
[2] In ml/24 h per 100 g BW.
[3] In ml/24 h per 100 g BW; * P<0.05 with respect to C.
[4] In ml/24 h per 100 g BW; * P<0.05 with respect to C; † P<0.05 with respect to S.
doi:10.1371/journal.pone.0043791.t001

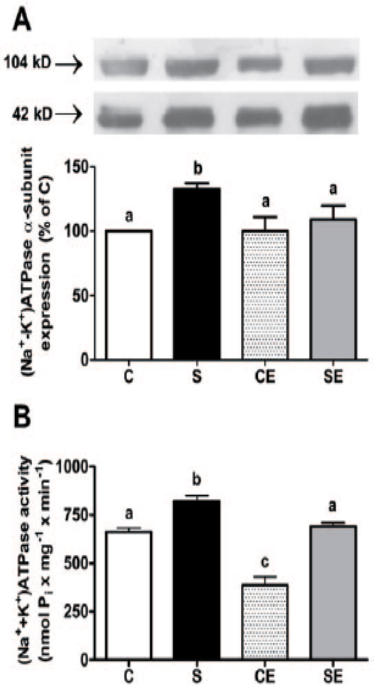


**Figure 2. Expression and activity of $(Na^+ + K^+)$ATPase in membranes of proximal tubule cells.** (A) upper and middle panels demonstrate representative immunoblots of $(Na^+ + K^+)$ATPase α-subunit (104 kD) and β-actin (42 kD), respectively. Lower panel: densitometric representation of α-subunit expression corrected by corresponding β-actin immunostaining (same lane); n = 8 (C and S) and n = 9 (CE and SE). Densitometric records were converted to percent values taken as 100% that of the C group obtained from the same nitrocellulose membrane. B: $(Na^+ + K^+)$ATPase activity. Results are means ± S.E.M.; n = 6 (C and S), n = 5 (CE) and n = 7 (SE). Different lowercase letters above the bars indicate statistical difference (P<0.05).
doi:10.1371/journal.pone.0043791.g002

## $AT_1$ and $AT_2$ receptors density

To investigate whether $Na^+$- and enalapril-induced changes in $Na^+$-dependent ATPases activities (Fig. 2 and Fig. 3) and kinase activities (see below) were due to alterations at the beginning of the RAS cascade, the expression of $AT_1$ and $AT_2$ receptors in proximal tubule membranes were analyzed in 90 day old rats. Maternal $Na^+$ overloading and enalapril treatment after weaning

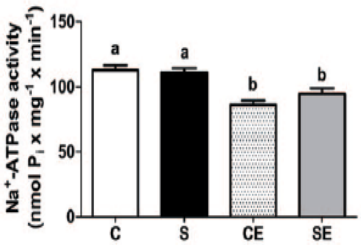


**Figure 3. Activity of the ouabain-insensitive $Na^+$-ATPase in membranes of proximal tubule cells.** Values are means ± S.E.M.; n = 26 (C), n = 23 (S), n = 22 (CE) and n = 21 (SE). Different lowercase letters above the bars indicate statistical difference (P<0.05).
doi:10.1371/journal.pone.0043791.g003

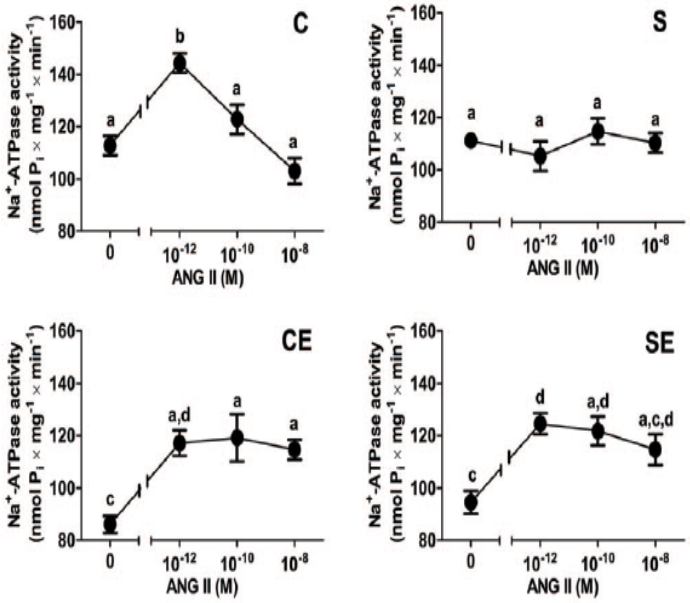


**Figure 4. Responsiveness of ouabain-insensitive $Na^+$-ATPase activity to Ang II.** Panels C (upper left) and S (upper right) correspond to the offspring from control and perinatally $Na^+$-overloaded mothers, respectively. Panels CE (lower left) and SE (lower right) correspond to the previous two groups treated with enalapril. Values are means ± S.E.M.; (n = 5–26 depending on the group and Ang II concentrations shown on the *abscissae*). Different lowercase letters above the circles indicate differences ($P<0.05$) calculated by comparing mean values within and among figures. Two or three letters above the same bar (a, d; a, c, d) relate to the fact that the corresponding mean value is not different from the others.
doi:10.1371/journal.pone.0043791.g004

had no effect (alone or combined) on $AT_1$ receptor expression (Fig. 5A). However, perinatal $Na^+$ overload programmed down-regulation of $AT_2$ receptors in adult offspring (S), an effect that was provoked to a comparable extent by enalapril treatment in non-programmed animals (CE). The more accentuated down-regulation observed in the SE group appeared to indicate that the effects of each treatment are additive.

## PKC and PKA activities and impact of specific inhibition of PKA

Fig. 6A demonstrates that PKC activity in renal membranes was increased by 50% in the S group compared with the C group. Enalapril treatment after weaning did not affect PKC activity in control rats (CE) but promoted a strong decrease in prenatally $Na^+$ overloaded rats (SE). PKA was increased by more than 100% in S rats (Fig. 6B), and treatment with enalapril reduced PKA activity in programmed and non-programmed groups to comparable very low levels (CE and SE).

Fig. 7 presents data regarding the influence of inhibition of PKA on $Na^+$-ATPase activity in the various offspring groups. When the assays were supplemented with PKAi to shut down PKA, the most remarkable effect was a greater than 65% increase in the activity of the pump in the S group.

## Renal TBARS, creatinine clearance and proteinuria

Fig. 8A demonstrates that perinatal $Na^+$ overloading markedly increased renal TBARS in the S group. Administering enalapril to non-programmed rats (CE group) increased TBARS, but did not augment cumulatively the TBARS levels in programmed rats (SE group). With respect to creatinine clearance (Fig. 8B), there was no modification in S rats, and the most striking effect – a significant decrease – was observed in the SE group, with a less pronounced effect in CE animals. Enalapril decreased proteinuria regardless of whether rats were exposed to high levels of $Na^+$ during prenatal and lactational periods.

## Immunohistochemical and morphometrical data

Fig. 9 demonstrates that macrophage infiltration increased in the renal cortical and medullary regions in the S, CE and SE groups. Collagen density (Fig. 10) increased by more than 150% in the cortex and by 60% in the medulla of rats in the S group, an effect that was reversed to a great extent (cortex) or completely (medulla) by enalapril administration. Enalapril alone increased collagen deposition (CE) but to a lower extent than that observed with cortical ED1 antigen (compare left graph bars in Figs. 10 and 9).

Morphometrical analysis demonstrated that the areas of glomeruli, glomerular capillary tufts and urinary spaces within glomeruli, remained unaltered independent of the treatment administered (data not shown).

## Plasma levels of Ang I

Plasma levels of Ang I were measured to investigate whether perinatal $Na^+$ overload programs alterations in the systemic RAS of the adult offspring. The data depicted in Fig. 11 demonstrate that perinatal $Na^+$ overloading programmed a decrease of more than 60% in circulating Ang I in adult offspring (S). In enalapril-treated rats (CE and SE), Ang I levels were comparable to those in the S group.

## Ang II determinations in renal cortex

The number of Ang II-positive cells in the tubulointerstitial region increased by 25% with respect to controls as a result of perinatal $Na^+$ overload (Fig. 12, compare C and S), and enalapril treatment caused a comparable decrease in the CE and SE groups. When the number of Ang II-positive cells was counted in

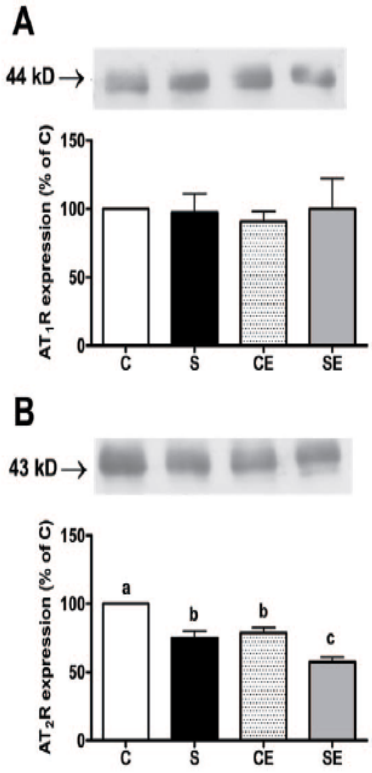


**Figure 5. $AT_1$ receptors and $AT_2$ receptors expression in membranes of the proximal tubule cells.** $AT_1$ (A) and $AT_2$ (B) receptores. Upper panels: representative immunodetections. Lower panels: densitometric representations of receptor immunosignals corrected for protein loading (ponceau red) after immunoprecipitation. Values are means ± S.E.M.; (n = 5 for $AT_1$; n = 3–5 for $AT_2$). C values were taken as 100% and those from S, CE and SE groups in the same gel were expressed as a percentage of C (n = 3–5 for $AT_2$; n = 5 for $AT_1$). Different lowercase letters above bars indicate statistical differences ($P<0.05$).
doi:10.1371/journal.pone.0043791.g005

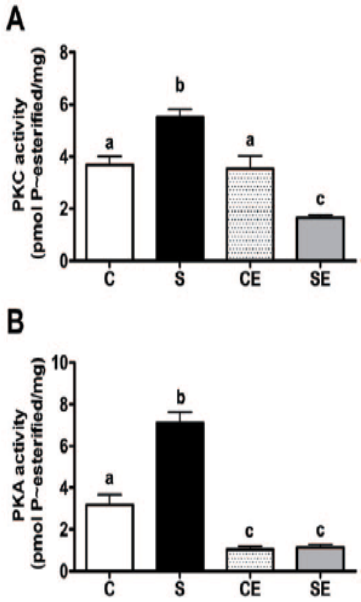


**Figure 6. PKC activity and PKA activity in proximal tubule cell membranes.** PKC activity (A) and PKA activity (B). Values are means ± S.E.M. (n = 3 for PKC and n = 4–5 for PKA). Different lowercase letters above bars indicate statistically different values ($P<0.05$).
doi:10.1371/journal.pone.0043791.g006

glomeruli, the number was the same regardless of whether the animals were submitted to perinatal $Na^+$ overload or not, or to enalapril treatment after weaning (Fig. 12).

## Discussion

The present study describes results that partially elucidate renal molecular mechanisms underlying alterations in active $Na^+$ transport across proximal tubule epithelium and local Ang II pathways in young adult offspring, which were programmed due to maternal $Na^+$ overload during gestation and lactation. Furthermore, this research demonstrates that short-term treatment with enalapril after weaning can reprogram much of the $Na^+$ overload-induced renal alterations in progeny, but paradoxically, in some cases this treatment can mimic or accentuate the impact of $Na^+$ overload.

Permanent up-regulation of the $Na^+$ pump responsible for the majority of $Na^+$ reabsorption in proximal tubules, ($Na^+$+$K^+$)ATPase [19], is evident in Fig. 2. This may be the result of erroneous adaptive signaling processes involving nuclear and cytosolic factors and enzymes such as PKC [33,34], activated as a consequence of low level apical $Na^+$ entry. This incorrect signal could be elicited by the retraction of brush border $Na^+$ transporters, viewed as a response to increased luminal $Na^+$ during $Na^+$ overloading and augmented filtered $Na^+$ [35]. Up-regulated PKC (Fig. 6A) could be responsible for these alterations by direct regulatory phosphorylation of the α-catalytic subunit, impacting enzyme turnover [36]. In addition, programmed up-regulation of PKA (Fig. 6B), which stimulates ($Na^+$+$K^+$)ATPase in several tissues [37,38], may be another incorrect signal that accounts for direct and permanent stimulation of ($Na^+$+$K^+$)ATPase above normal levels. With respect to expression of ($Na^+$+$K^+$)ATPase, PKA promotes targeting of pump units in basolateral membranes of proximal tubules [38,39], which was evident in the adult offspring during this study (Fig. 2A). However, despite this plausible explanation, an opposite mechanism could be responsible for the up-regulation of ($Na^+$+$K^+$)ATPase: the stimulus resulting from a long term increased apical $Na^+$ entry. This alternative view is supported by the observation that high $Na^+$ intake increases removal of fluid from the lumen of rat proximal tubules [40], an indication of augmented primary $Na^+$ flux across the apical membrane.

The ouabain-resistant, furosemide-sensitive $Na^+$-ATPase has been cloned and purified recently [41] and, in kidney, it is considered responsible for fine tuning of $Na^+$ reabsorption in proximal tubules [20,42]. $Na^+$-ATPase, which is modulated *in vitro* by hormones and autacoids that participate in the physiological regulation of extracellular fluid [42,43], was unaltered in the membranes of animals in the S group (Fig. 3). A constitutive increase in activity was expected facing the up-regulation of PKC (Fig. 6A), which plays a role in its physiological activation [20]. However, the significant counteracting increase in PKA activity could negate the influence of PKC [43,44] in rats programmed by $Na^+$ overload. This is an idea that receives support from the observation that the PKA inhibitor peptide elicited a pronounced elevation of $Na^+$-ATPase (Fig. 7). Therefore, the effect of PKA action on $Na^+$-ATPase was different from our previous results [44] and those from other laboratories [42]. However, the stimulatory

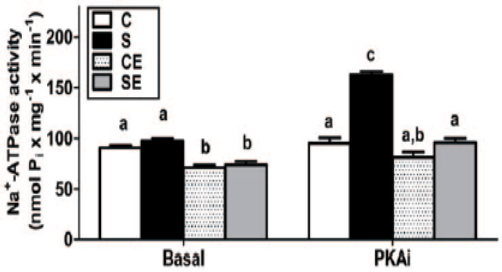


**Figure 7. Ouabain-insensitive $Na^+$-ATPase activity measured in the absence or presence of $PKAi_{5-24}$ peptide.** Absence (basal condition, left) or presence (right) of $PKAi_{5-24}$ peptide. The results depicted in Fig. 3 (basal) are presented and statistically analyzed in conjunction with those obtained in the presence of PKAi (n = 7). Results are means ± S.E.M. Different lowercase letters above bars indicate statistical differences ($P<0.05$). Two letters above the same bar (a, b) relate to the fact that the corresponding mean value is not different from others.
doi:10.1371/journal.pone.0043791.g007

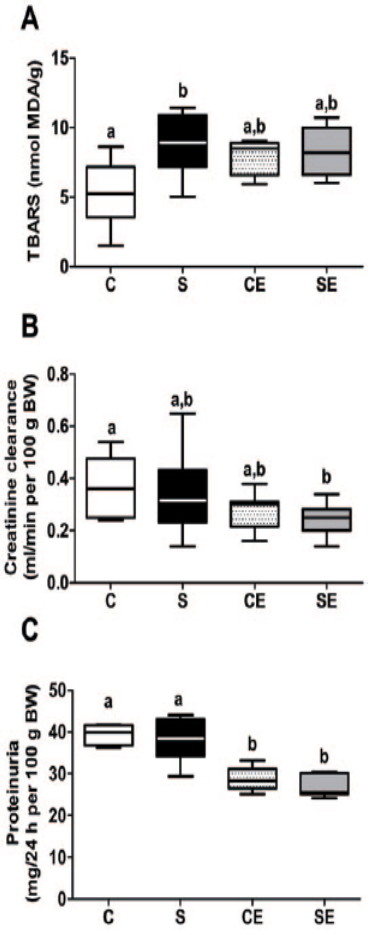


**Figure 8. Thiobarbituric acid reactive substances (TBARS) in renal tissue, creatinine clearance and proteinuria.** TBARS (A), creatinine clearance (B) and proteinuria (C). The box plots in the three panels present the median, minimum and maximum values; n = 8–12 (A), n = 13–16 (B) and n = 7–8 (C). Different lowercase letters above bars indicate statistical differences ($P<0.05$). Two letters above the same bar (a, b) relate to the fact that the corresponding mean value is not different from others.
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effect of physiological Ang II concentrations on $Na^+$-ATPase correlates with PKC [20,43], and the loss of the normal biphasic influence of the peptide (Fig. 4, upper right panel) could be due to the imbalance between PKC and PKA. It is of special interest in the context of the present work that a recent phosphoproteomic-based study demonstrated that several effector proteins can be selectively phosphorylated in response to Ang II [45].

The loss of the inhibitory effect of high Ang II concentrations in animals from the S group (Fig. 4) could be due to a programmed decrease in the expression of $AT_2$ receptors (Fig. 7A), the first step in the Ang II-associated cascade that culminates in the inhibition of $Na^+$-ATPase caused by high concentrations of the peptide [46]. Despite the lower expression of $AT_2R$ in kidney, there is growing evidence about their crucial role in renal physiological and pathological processes. For example, beneficial effects on renal injury achieved with $AT_1R$ blockade are potentiated by Ang II effects transduced through $AT_2R$, in a model where collagen deposition is significant [47], as in our model (Fig. 10).

Perinatally $Na^+$ overloaded adult offspring presented with increased macrophage infiltration in the whole kidney, and collagen deposition in the cortex that was not counteracted by enalapril treatment (Figs. 9 and 10). However, it is striking that enalapril *per se* augmented the inflammatory response, and promoted modest but significant collagen deposition, macrophage infiltration and TBARS increment, as ACE inhibition ameliorates several pathological indicators including local oxidative stress in uremic rats [48]. Decreased proteinuria (Fig. 8C) was in accordance with the known beneficial actions of RAS blockade. Despite the experimental contradiction, it is possible that a common factor, increased ROS production, is involved in these structural alterations programmed in perinatally $Na^+$ overloaded rats. High oxidant production has been correlated with renal inflammation indicators [7,49,50], and consequently it is plausible that ROS contributed to macrophage infiltration and collagen deposition as a legacy imprinted by enalapril administration in the short window of 21 days after weaning. Regarding the latter process, the significant increase in Ang II levels in the cortical tubulointerstitium of the S group (Fig. 12) could be another key factor that contributed to collagen deposition in association with ROS. Recently, it was demonstrated that Ang II-induced ROS play a central role in the signaling cascade that accentuates collagen deposition in renal tissue, which can culminate in fibrosis [51].

At this point a question emerges. How would enalapril induce increased ROS production? It is likely that the drug impacted two physiological processes, oxidant production (Fig. 8A) and the glomerular filtration rate (GFR), at the same time. Its considerable

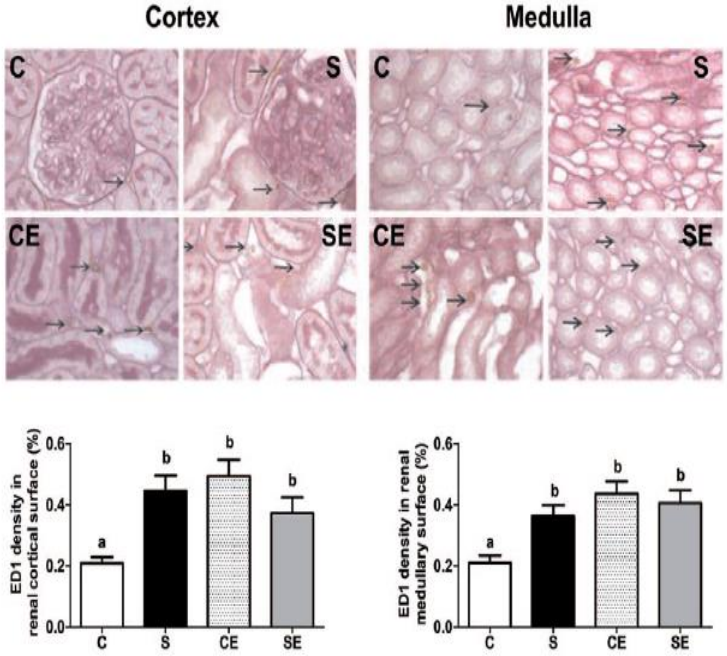


**Figure 9. Macrophage infiltration of cortical and medullary regions evaluated by immunostaining of ED1 antigen.** Upper and middle panels are representative fields (×400) of cortex and medulla from C, S, CE and SE experimental groups. Arrows point to ED1 positive cells. Lower panels present percentage values of ED1 surface density per field in cortex (left) and medulla (right). Different lowercase letters above bars indicate statistically different values ($P<0.05$).
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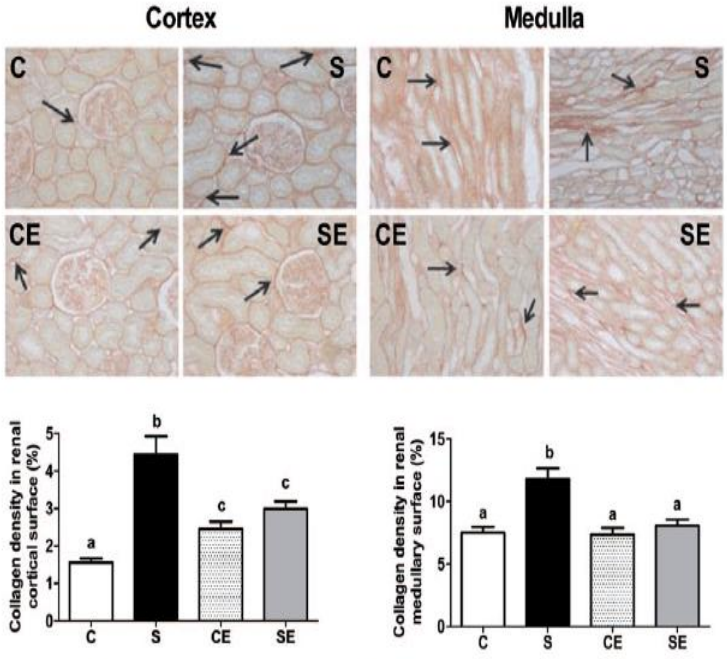


**Figure 10. Collagen staining of cortical and medullary regions.** Upper and middle panels are representative fields (×200) of cortex and medulla from the C, S, CE and SE experimental groups. Arrows point to collagen deposition. Lower panels present percentage values of collagen per field in cortex (left) and medulla (right). Different lowercase letters above bars indicate statistically different values ($P<0.05$).
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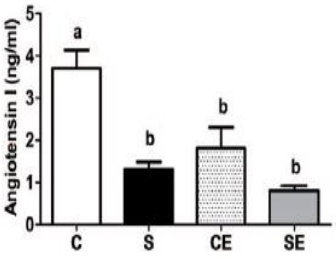


**Figure 11. Plasma levels of Ang I.** Values are means ± S.E.M. of 6–8 determinations using different rats, carried out in duplicate. Different lowercase letters above bars indicate statistical differences in mean values ($P<0.05$).
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impact on creatinine clearance (Fig. 8B), through a common mechanism of reducing blood pressure during administration after weaning (Fig. 1, empty points above the horizontal line) has been demonstrated. Reduced blood pressure is likely to have led to lower renal blood flow and renal hypoxia. Programming vasodilatation of the efferent arteriole as a consequence of local RAS inhibition and decreased local Ang II (Fig. 12), could explain the reduction of GFR during adult life (Fig. 8B).

Interestingly, enalapril provoked a decrease in $AT_2$ receptor expression (Fig. 5B), a side effect that reinforces the idea of simultaneous actions of the drug beyond its action in reprogramming ($Na^+$+$K^+$)ATPase expression and turnover (Fig. 2). The additive effects of perinatal $Na^+$ overload and enalapril treatment on $AT_2$ receptor expression (SE, Fig. 5B) clearly demonstrates there are separate pathways in which RAS participate, affecting renal molecular programming and reprogramming. The effects of enalapril treatment after weaning support the view that renal programming continues after nephrogenesis is complete [17]. The influences of $Na^+$ overload and enalapril treatment are not additive in terms of effects on renal TBARS (Fig. 8A). Therefore, they are likely to impact on a common final enzymatic target in tubule cells, thereby impairing ROS detoxification.

Perinatal $Na^+$ overload programmed two opposite alterations regarding RAS during adulthood. First, it strongly depressed levels of circulating Ang I, probably as a result of inhibiting systemic renin release that persisted in adult life. Indeed, plasma renin activity is lowered during high $Na^+$ overload [52]. Second, high perinatal $Na^+$ programmed elevated enalapril-sensitive levels of Ang II in cortical tubulointerstitium. The programming of enalapril-sensitive increased levels of local Ang II caused by $Na^+$ overload, impacted ($Na^+$+$K^+$)ATPase and the ouabain-resistant $Na^+$-ATPase as demonstrated in Figs. 2, 3 and 4, thereby contributing to increased $Na^+$ reabsorption across proximal tubular epithelium, as is the case during continuous infusion of Ang II [53].

However, perinatally $Na^+$-overloaded rats did not present with modifications in the levels of local Ang II in glomeruli (Fig. 12), and this correlates with a lack of alterations in the glomerular capillary tufts and the Bowman's capsule (data not shown). These negative results are important, as they reinforce the idea that local variations in Ang II levels and associated signaling pathways can be ascribed to structural and functional alterations in a renal tissue impacted by $Na^+$ overload programming.

Finally, despite the important structural and functional renal alterations induced by perinatal $Na^+$ overload, the rats were not

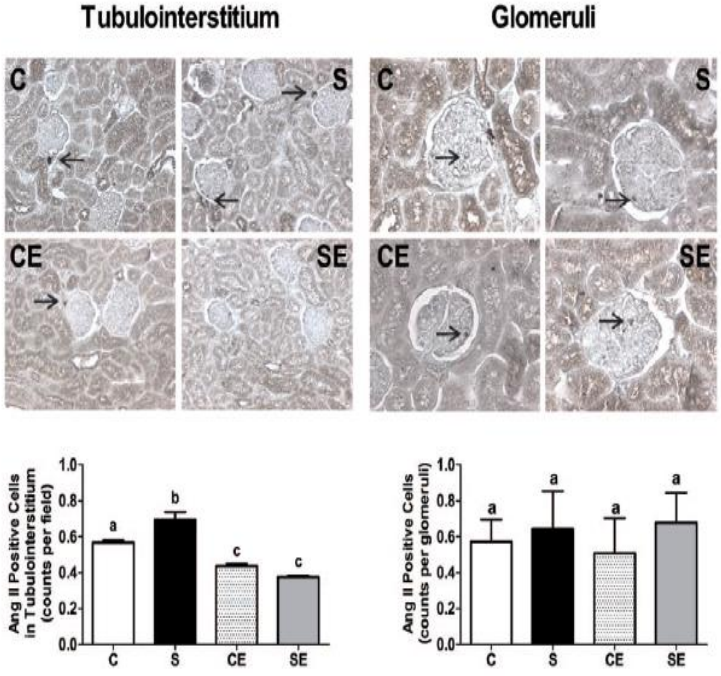


**Figure 12. Immunodetection of Ang II in the renal cortex.** Upper and middle panels are representative fields demonstrating Ang II positive cells in the C, S, CE and SE groups counted in tubulointerstitium (×100) and glomeruli (×200), as indicated at the top of the figure. Arrows point to Ang II positive cells. Lower panels present graphic representations of counting (means ± SE). Different lowercase letters above bars indicate statistical differences ($P<0.05$). Each mean ($n=5$) resulted from counting 60 fields in each kidney.
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hypertensive at 90 days old. Lower levels of circulating Ang I (Fig. 11) in the S group – as the result of an impact in systemic RAS – could be responsible for these low pressure levels. Salt loading via drinking water, as used in the present study, differs from high salt food intake by humans in that there was no scope for compensatory water intake and so, this method of salt loading may be a limitation. Nevertheless, it is clear that the ensemble of programmed alterations described herein could be the basis for the expansion of extracellular compartments, the increase in bodily $Na^+$ content [22] and, possibly, the onset and maintenance of hypertension in older rats. Longer term experiments are now carried out to test this hypothesis. However, early treatment with enalapril reprogrammed and reduced the majority of alterations with regards to $Na^+$ reabsorption and local Ang II levels, but side effects, particularly those associated with increased lipid peroxidation, down-regulation of $AT_2$ receptors, and significant modifications to kinase activities, GFR, macrophage infiltration and cortical collagen deposition, could have an overall negative impact on renal function in older rats.

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## Author Contributions

Conceived and designed the experiments: EVC LDV-F AV ADOP. Performed the experiments: EVC LDV-F PAS WSN RSA FSTO RL. Analyzed the data: EVC LDV-F PAS WSN RSA FSTO RL AV ADOP. Contributed reagents/materials/analysis tools: AV ADOP. Wrote the paper: EVC LDV-F PAS RL AV ADOP.

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# ANEXO II

# Alpha-Tocopherol Supplementation During Lactation or Shortly After Weaning Impairs Cardiac Development and Increases Blood Pressure at Adult Life in Rats[1,2]

**Running title** α-tochopherol programming hypertension

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**Word count (abstract through references): 7,458**

**Number of figures:** 8

**Number of tables:** 2

**Abbreviations used:** ACEI, angiotensin converting enzyme inhibitor; AngII, angiotensin II; BRS, baroreflex sensitivity; BW, body weight; CTL, rats prenatally provided with tap water and treated with α-tocopherol along lactation; CTW, rats prenatally provided with tap water and treated with α-tocopherol after lactation, for 3 weeks; CV, rats prenatally provided with tap water and treated with α-tocopherol vehicle along lactation; GFR, glomerular filtration rate; GSH, reduced glutathione; HR, heart rate; IU, international unit; L, lactation; MDA, malondialdehyde; NADPH, β-Nicotinamide adenine dinucleotide 2′-phosphate reduced; $O_2^-$, superoxide anions; PMCA, plasma membrane $Ca^{2+}$-ATPase; PMSF, phenylmethanesulfonyl

fluoride; RLU, relative light units; ROS, reactive oxygen species; SERCA, sarcoplasmic reticulum $Ca^{2+}$-ATPase; SHR, spontaneously hypertensive rats; STL, rats prenatally provided with 0.3 M NaCl solution and treated with α-tocopherol along lactation; STW rats prenatally provided with 0.3 M NaCl solution and treated with α-tocopherol after lactation, for 3 weeks; SV, rats prenatally provided with 0.3 M NaCl solution and treated with α-tocopherol vehicle along lactation; T, α-tocopherol; TCA, trichloroacetic acid; tcSBP, tail-cuff systolic blood pressure; $U_K{}^+V$; MAP, mean arterial pressure; $U_{Na}{}^+V$, urinary $Na^+$ excretion; $U_{Prot,}$ 24-h proteinuria; W, weaning.

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[2]The authors have no conflicts of interest.

**Abstract**

α-Tocopherol (T) supplementation during lactation increases blood pressure at later life, in part due to functional changes in the kidneys. Beside the kidneys, the present study investigated the effects of T also on the heart. Reactive oxygen species (ROS) and angiotensin II (AngII) were investigated as being the ones accountable for functional changes in the two organs. T was administered during two short periods: along lactation and throughout the first 3 weeks after weaning. Male Wistar rats, offspring of mothers maintained on tap water, control dams, or on a 0.3 M NaCl solution were used. T was administered to mothers daily (0.35g/kg, by gavage) during lactation or to the offspring for the first 3 weeks after the weaning. Tail-cuff systolic blood pressure was measured from age of 150 to 210 days. Mean arterial pressure and heart rate were measured before and during AngII infusion (100 ng/kg/min, iv). Superoxide anion production was measured by lucigenin-produced chemiluminescence, before and after adding NADPH in the left ventricle and kidneys. β1-adrenergic receptor expression, plasma membrane $Ca^{2+}$-ATPase and sarcoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) activities were measured in the left ventricle. T over lactation led to increased blood pressure, hyporesponsiveness to AngII-induced hypertension and cardiac atrophy in offspring prenatally provided with tap water or NaCl solution. β1-adrenoreceptor and SERCA alterations were underlying cardiac functional alterations. T supplementation after weaning had a pro-oxidative effect in the left ventricle and led to AngII-induced tachycardia in rats prenatally exposed to high sodium. Despite cardiac functional alterations, ROS were unchanged in heart or kidneys of animals treated with α-tocopherol during lactation. In contrast, increased ROS seems to account for cardiac functional alterations in animals treated with α-tocopherol after weaning. In summary, vitamin E supplementation over early development might be an etiological agent to later hypertension.

**Key words:** α-Tocopherol supplementation, fetal development, cardiac function, blood pressure, reactive oxygen species, renal function.

## Introduction

Antioxidant vitamins, such as vitamin E, as well as other micronutrients, have been used as nutritional supplementation during the periconceptional period to lower malformation risks (1). Moreover, antioxidants have been applied as an attempt to prevent preeclampsia and other adverse maternal and perinatal outcomes (2). Experimental data has shown beneficial effects to offspring when α-tocopherol, the most active form of tocopherols, is supplemented during their whole life (3) or only during lactation (4–6). The positive outcomes of α-tocopherol are attributed to its antioxidant action in the kidneys (3), which is capable of changing the course of hypertension, as well as recovering $Na^+$ transport in the proximal tubule of rats (4,6). Albeit its beneficial effects, it has been shown that α-tocopherol is not inactive in the condition of healthy state, i.e. in control rats (5, 6), since it leads to increased blood pressure (6).

Heart and kidneys are natural organs that regulate blood pressure. Cardiomyocyte development in rats continues over the postnatal period, when proliferation stops between days 3 and 4, and cardiac tissue switches to binucleation up until day 12 (7). Thereafter, it develops hypertrophy. Reactive oxygen species (ROS) have been described as acting on cardiac differentiation and growth (8–10), while inhibition of NADPH oxidase has been shown to abolish ROS-induced cardiomyogenesis (11).

Renal development in rats covers the prenatal period, from day 12, and is prolonged throughout the $10^{th}$ postnatal day of life (12, 13). Indeed, some findings show that a short period of treatment even after weaning is capable of changing renal function in the long term. A brief treatment with perindopril, an angiotensin converting enzyme inhibitor (ACEI), of 2

to 6 weeks of life, is sufficient to change the course of hypertension in adult spontaneously hypertensive rats (SHR) (14). Enalapril, another ACEI, administered for a period of 3 weeks after weaning reduces activity of ($Na^+$+$K^+$) ATPase and $Na^+$-ATPase in the proximal tubule (15). Furthermore, enalapril (16), or exposure to a low-sodium diet (17) for a period of 3 weeks after weaning, prevents adult hypertension in rats exposed to undernutrition during prenatal life.

The renin-angiotensin system emerges as a potential candidate to mediate cardiovascular action of early administered α-tocopherol. α-Tocopherol during lactation increases AngII in the kidneys (6), augments protein kinase C (PKC) activity and increases AngII-induced $Na^+$ transporter activity in membranes of proximal tubules (4) at adult age.

This study tested the hypothesis that short and early supplementation with α-tocopherol, along two different periods, during lactation or for 3 weeks after weaning, may program cardiac and renal function. The study sought to investigate ROS and the role of AngII, as being accountable for functional changes. Furthermore, to change the condition of healthy state, part of the offspring evaluated were born from mothers maintained on a 0.3 M NaCl solution, instead of drinking water, over a period of 20 days before and along the pregnancy.

**Materials and Methods**

*Materials*. α-tocopherol, angiotensin II, Triton X-100, proteases inhibitor cocktail, lucigenin, thiobarbituric acid, β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), 5,5′-Dithiobis(2-nitrobenzoic acid), trizma, Folin & Ciocalteu's phenol reagent, bovine serum albumin, thapsigargin, HEPES, phenylmethanesulfonyl fluoride

(PMSF), adenosine 5′-triphosphate magnesium salt, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), acrylamide, N,N′-Methylenebis (acrylamide), N,N,N′,N′-tetramethylethylenediamine, ammonium persulfate, glycine, BIS-TRIS propane and ouabain octahydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Commercial kit for creatinine measurement (Labtest, Lagoa Santa, MG, Brazil). Pentobarbital was purchased from Cristália (Produtos Químicos Farmacêuticos, Itapira, SP, Brazil). Trichloroacetic acid (TCA), sucrose, EDTA, calcium chloride and potassium chloride were purchased from Vetec (Rio de Janeiro, RJ, Brazil). Nitrocellulose blotting membrane, Horseradish peroxidase-conjugated anti-rabbit IgG antibody and the ECL Prime Western blotting system were purchased from GE Healthcare (Buckinghamshire, UK). Rabbit polyclonal antibody against β1 adrenergic receptor (ab-64996) was purchased from Abcam (Cambridge, MA, USA). All other reagents were of the highest purity.

*Animal groups*. Experimental protocol followed the guidelines and had approval of the Committee for Experimental and Animal Ethics at the Federal University of Pernambuco (no. 23076.055063/2010-03).

Seventy-day-old female Wistar rats, weighing 200–250 g, were randomly assigned to a maternal control group or saline group. Until parturition, the maternal control group (n=8) had free access to tap water and the maternal saline group (n=8) received a 0.3 M NaCl solution (1.8% w/v). At age of 90 days, the animals were mated. At birth, litters were reduced to eight pups (including females that were used in other experiments). Only male offspring were employed for experimental protocol. The rats were maintained with a standard commercial rodent diet (Purina Agribands, Paulínia, SP, Brazil). The control group treated with vehicle (CV, n=10) was born from dams that consumed tap water during pregnancy and received daily maize oil (V, 1 ml/kg, by gavage) during lactation (L). The control group treated with α-tocopherol during lactation, the CTL group (n=12), was born from dams that

consumed tap water during pregnancy and received daily α-tocopherol (T, 0.35 g/kg, by gavage) during lactation. The same treatment was given to dams that drank NaCl solution throughout the pregnancy to obtain the groups SV (n=7) and STL (n=9). Furthermore, part of the offspring born from C and S mothers, whose treatment was maize oil during lactation, received α-tocopherol from weaning (W), at age of 21 days, until age of 42 days, the groups CTW (n=11) and STW (n=8), respectively.

*Blood pressure and parameters of cardiac and renal function.* Tail-cuff systolic blood pressure (tcSBP) was measured by plethysmography (IITC Life Science B60-7/16'', Life Science Instruments, Woodland Wills, CA) after animals were acclimated to the room and to the cages of containment and warming, at ages of 150, 180 and 210 days. Acclimation was performed for three days. The room was kept silent and maintained under temperature around 22°C. To measure tcSBP, the animals were warmed at 36.6°C. tcSBP was obtained as a mean from an average of 3 to 5 measurements, per rat, at each experiment. Creatinine clearance, 24-h proteinuria ($U_{Prot}$) and urinary $Na^+$ and $K^+$ excretion were also evaluated at each of the ages in which tcSBP was obtained. Metabolic cages (Tecniplast Gazzada Sarl, Buguggiate, Italy) were used to collect 24-h urine samples to measure proteinuria, creatinine and $Na^+$. Blood samples were obtained from the caudal artery for creatinine measurements. At age of 210 days, 48 h after completion of the metabolic studies, animals were anesthetized using pentobarbital (60 mg/kg, ip) and surgically prepared for direct measurement of mean arterial pressure (MAP) and heart rate (HR) before and after AngII infusion (100 ng/kg/min, iv). The femoral artery was catheterized and connected to a blood pressure transducer (SP 844, MEMSCAP Inc, Durham, USA), assembled to a data acquisition unit (Power Lab, 8/30, ML866, ADInstruments, Australia), while the right jugular vein was catheterized for AngII infusion (11 plus, Harvard Apparatus, Holliston, MA, USA). MAP and HR were continuously

registered for 20 min, by using a Lab Chart software (7.3.4 version, ADInstruments). Baroreflex sensitivity (BRS) was calculated from the ratio AngII-induced change in HR/ AngII-induced change in MAP. Thus, BRS = Δ HR/ Δ MAP (18). Yet under anesthesia, the hearts and kidneys were withdrawn, rapidly weighed and flash frozen in liquid nitrogen before keeping them at – 80 °C. The rats were euthanized by exsanguination.

*Expression of β1-adrenoreceptor in left ventricle.* The left ventricle was homogenized in RIPA buffer in a proportion of 1mL:g of tissue. Tissues were homogenized into ice bath, using a tissue grinder, coupled to a rotor IKA RW20, at 1,200 rpm for 2 min. RIPA buffer contained: 50mM Tris, 150mM NaCl, 1mM EDTA, 1% triton X-100, 1%deoxycholate, 1% SDS, 1mM PMSF. Aliquots of 80 µg of protein were separated in 10% SDS-PAGE gel, and then transferred to a nitrocellulose membrane at 350 mA. First, non-specific binding was prevented by incubating the membranes in 5% BSA (diluted in Tris buffered saline solution containing 0.1% Tween 20, TBS-T) at room temperature for 1 hour. Sequentially, membranes were probed using rabbit primary antibody against a β1-adrenoreceptor, diluted in 5% BSA in TBS-T (1:10,000) for 2 hours, at room temperature, and incubated for 1 hour at room temperature with horseradish peroxidase-labeled antibody anti-rabbit IgG diluted in TBS-T (1:20,000). The membranes were washed in TBS-T after each antibody probe. Finally, the protein blots were visualized using a chemiluminescence kit. Densitometric analyses were performed using Scion Image for Windows (version Alpha 4.0.3.2, Scion Corporation) and normalized according to the protein load, visualized at Ponceau Red staining.

*Left ventricle plasma membrane $Ca^{2+}$-ATPase and sarcoplasmic reticulum $Ca^{2+}$-ATPase.* Plasma membrane $Ca^{2+}$-ATPase (PMCA) and sarcoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) activity were evaluated in the left ventricle, as previously described (19), with some

modifications. Samples were thawed into ice bath to prepare the homogenate by using a solution containing 250 mM sucrose, 10 mM HEPES-Tris (pH 7.4), 2 mM EDTA, 0.15 mg/l trypsin inhibitor (type IIS) and 1 mM PMSF, in a proportion of 1g tissue:4 ml solution. The measurement of ATPases activities was developed by evaluating inorganic phosphate ($P_i$) released from ATP. Samples were added to a solution containing 50 mM BIS-TRIS propane, 10 mM $NaN_3$ and 5 mM MgCl, for 10 minutes, at 37ºC. Total $Ca^{+2}$-ATPase activity corresponded to the $P_i$ released in the absence and in the presence of 2 mM EGTA. SERCA activity was represented by the difference between ATP hydrolysis in the absence and in the presence of 3 µM thapsigargin, while PMCA activity was estimated by the residual $Ca^{2+}$-ATPase activity. Thus, PMCA is the result of the total $Ca^{+2}$-ATPase activity minus the thapsigargin sensitive $Ca^{2+}$-ATPase activity. The assays were performed intriplicate.

*Superoxide anions in heart and kidneys.* Superoxide anions ($O_2^-$) were measured according to a previous report (20). Tissues were homogenized into RIPA buffer (described above). Protease inhibitor cocktail was added to RIPA. The ratio was 1 g tissue to 7 ml RIPA. The homogenate was centrifuged by using a refrigerated centrifuge (NT805, Novatecnica, Piracicaba, SP) at 12,000 ×*g*, 4°C for 12 min, to use the supernatant. A saline phosphate buffer (PBS, pH 7.4) was added to the supernatant, in a ratio of 10ml:1ml. A Luminometer (Varioskan Flash, Thermo Scientific, Vantaa, Finland) was used to measure the chemiluminescence for 10 min, at 37°C, after adding 10µM lucigenin. Chemiluminescence was also measured for the same amount of time in the presence of 100µM NADPH. The assays were performed in triplicate.

Total ROS was quantified by immunoassay using a commercial kit (My Biosource, San Diego, CA, USA). The kidney samples were thawed and homogenized into 0.02 M PBS, pH 7.4, 4°C, at a proportion of 10 ml per gram of tissue. Sequentially, the homogenates were

submitted to ultrasonication for 10 min and centrifuged at 1,500 ×*g* for 15 minutes, at 4ºC. The supernatant was used for the ROS measurement, according to manufacturer guidelines. The results were calculated using a set of calibration standards, which accompanied the kit, and corrected by total protein content. The assays were performed in duplicate.

Additionally, levels of malondialdehyde (MDA) and reduced glutathione (GSH) were measured. For this, the tissues were homogenized in 150 mM KCl, 1g tissue:5 ml solution. The levels of MDA were measured according to previous reports (21) with some modifications. For the standard curve, 1,1,3,3-tetraethoxy-propane was used. Levels of GSH were assessed as non-protein sulfhydryl groups (22). L-cysteine was used for the standard curve. Both the MDA and GSH results were corrected for protein concentration. The assays were performed in duplicate.

*Analytical methods.* Protein in tissue samples and urinary protein was measured using the Folin phenol method (23). Urinary $Na^+$ and $K^+$ were measured using an electrolyte analyzer (AVL 9180, Roche Diagnostics GmbH, Mannheim, Germany). Serum creatinine was determined using a commercial kit.

*Statistical Analyses.* The results are expressed as mean ± SEMs. Differences between experimental groups were analyzed using two-way ANOVA followed by Bonferroni test, while the differences between Ang II-stimulated MAP and HR versus basal conditions were analyzed by paired Student's *t* test. One sample *t* test was employed to contrast the β1-adrenoreceptor expression of the several groups against the CV group. The statistical analyses were performed using GraphPad Prism 5 (Version 5.01, GraphPad Software, Inc.).

**Results**

*Body weight development from birth to adulthood and organ weight index at adulthood.* At birth, C and S groups presented the same body weight (CV = 6.4 ± 0.2 and SV = 6.6 ± 0.3 g). Maternal treatment with α-tocopherol during lactation did not affect body weight (BW) at weaning, since CV, CTL, SV and STL groups maintained similar BW (CV = 48 ± 1; CTL = 49 ± 1; SV = 52 ± 1; STL = 50 ± 1 g). However, the CTL group showed a lower BW ($P < 0.05$) than the CV group, at age of 90 and 120 days (Fig. 1A),while all saline groups showed the same BW development along the treatment (Fig. 1B). Although saline rats had unchanged BW at birth and weaning, the SV group exhibited higher BW than the CV group (Fig. 1C).

At age of 210 days, CTL and STL presented a lower ($P < 0.05$) cardiac weight index than their respective control groups CV and SV, respectively (Fig. 2A). On the other hand, the renal weight index was similar among all groups (Fig 2B).

*Cardiovascular functional parameters.* tcSBP was measured monthly in conscious rats, from 150 to 210 days of life, as shown in Fig 3. Compared to the CV group, the CTL group showed higher tcSBP levels at 150 and 210 days (12 and 10 mmHg, respectively, $P < 0.01$). Similarly, the CTW group showed higher tcSBP than the CV group, at 210 days (8 mmHg, $P < 0.05$). The saline groups showed a profile that was quite opposite to that of control groups. SV showed higher tcSBP than CV, at 150, 180 and 210 days (19, 14 and 23 mmHg, respectively, $P < 0.001$). On the other hand, STL showed lower tcSBP than SV, at each moment evaluated (15, 15 and 14 mmHg, respectively $P< 0.001$). STW exhibited lower levels of tcSBP than SV, at 150 days (8 mmHg, $P< 0.01$).

MAP measured in anesthetized rats, at age of 210 days, is shown in Fig. 4A. CTL showed higher MAP than CV. Differently from tcSBP observed in conscious rats, MAP was unaltered in SV compared to CV. Furthermore, STL and STW showed higher ($P< 0.05$) levels of MAP than the SV group.

The effectiveness of AngII infusion to augment MAP was abolished in the CTL and STL groups. They exhibited a statistically insignificant increment in MAP compared to their respective control groups, CV and SV, respectively (Fig. 4B). Basal HR, measured before AngII infusion, was similar among groups, except for the CTL group, which showed higher HR than the CV group ($P< 0.05$, Fig. 4C). Under AngII infusion, all groups, except STW, showed similar declines in HR (Fig. 4D, $P< 0.05$). Under AngII infusion, the STW group presented elevation in HR ($P< 0.01$). In light of this profile, all groups showed unchanged baroreflex sensitivity, while STW showed augmented ratio AngII-induced change in HR/ AngII-induced change in MAP, which points to diminished baroreflex sensitivity (Fig. 4E).

*Cardiac expression of β1-adrenoreceptor.* Expression of β1-adrenoreceptor protein in the left ventricle is shown in Fig. 5. CTL and STL presented lower ($P < 0.001$) expression of this receptor than their respective control groups, CV and SV, respectively. Meanwhile, the β1-adrenoreceptor protein expression was similar between CTW and CV, as well as between STW and SV. Moreover, pre-natal saline treatment did not affect this receptor; SV and CV were similar. On the other hand, STW had a higher β1-adrenoreceptor expression ($P< 0.05$) than CV or CTW.

*Cardiac $Ca^{+2}$-ATPase activities: SERCA and PMCA.* SERCA and PMCA activities evaluated in the left ventricle homogenates of rats at age of 210 days are shown in Fig. 6A. CTL presented elevation ($P< 0.05$) in PMCA and reduction ($P <0.05$) in SERCA activity. On the other hand, STL showed increased ($P< 0.01$) SERCA activity and unaltered PMCA activity. To illustrate the profile of activity from these enzymes, Fig. 6B shows the ratio PMCA:SERCA. The figure shows that this ratio was elevated ($P< 0.001$) in the CTL group

compared to the CV group, and that it was unchanged in the STL group compared to the SV group.

$O_2^-$ *in heart.* $O_2^-$ production in the left ventricle of rats, at age of 210 days, is shown in Fig. 7. Before adding NADPH, the basal $O_2^-$ production was increased ($P < 0.001$) in CTW compared to CV and reduced ($P< 0.05$) in SV compared to CV (Fig. 7A). NADPH-stimulated $O_2^-$ production was increased ($P< 0.001$) in CTW compared to CV and also in STW compared to SV (Fig. 7B). However, taking into account the increased basal $O_2^-$ levels in the CTW group, only the STW group showed an augmented difference between the levels observed in the presence and absence of NADPH (Fig. 7C).

*Oxidative stress markers in kidneys*. Table 1 shows oxidative stress markers evaluated in the kidneys of 210 day old rats. Levels of malondialdehyde, GSH, total ROS levels, and the $O_2^-$ measured by lucigenin chemiluminescence, basal and NADPH-dependent, are shown in the table. None of these markers were altered by α-tocopherol or saline treatments, except for the NADPH-dependent $O_2^-$ production, which was 31% lower ($P < 0.05$) in STW compared to the SV group.

*Renal function: Urinary $Na^+$ and $K^+$ excretion, proteinuria and creatinine clearance.* Urinary $Na^+$ excretion ($U_{Na^+}V$) at age of 150, 180 and 210 days is shown in Fig. 8A. At 150 days, the CTW and SV groups showed lower ($P< 0.05$) $U_{Na^+}V$ than the CV group. At 180 days, CTL presented lower ($P< 0.001$) $U_{Na^+}V$ than the CV group. No differences were observed among the groups in $U_{Na^+}V$ at age of 210 days.

Urinary $K^+$ excretion ($U_{K^+}V$) is shown in Table 2. At age of 150 days, $U_{K^+}V$ was similar among CV, CTL and CTW and also among SV, STL and STW groups. However, it

was lower ($P< 0.05$) in SV than in the CV group. At age of 180 days, $U_K{}^+V$ was lower ($P< 0.001$) in CTL and CTW than in the CV group. It was also lower in STL than in the SV group. At age of 210 days, $U_K{}^+V$ was similar among all groups.

The glomerular filtration rate (GFR), measured as creatinine clearance is shown in Fig. 8B. At age of 150 days, CTW showed lower ($P< 0.001$) GFR than the CV group. GFR was lower ($P< 0.01$) in SV than in the CV group. Moreover, the STW group showed lower ($P< 0.01$) GFR than the SV group. At age of 180 days, CTL and CTW groups showed lower GFR than the CV group, while SV, STL and STW presented the same values among each other and SV showed lower values ($P< 0.001$) than the CV group. At age of 210 days, GFR was similar among all groups.

24-h proteinuria ($U_{Prot}$) was similar among all groups at age of 150 and 210 days (Table 2). At age of 180 days, CTW showed lower ($P< 0.05$) values of $U_{Prot}$ than the CV group. $U_{Prot}$ in SV was lower ($P< 0.05$) than in the CV group, while in STL it was lower ($P< 0.05$) than that in STW (Table 2).

**Discussion**

Independently from treatment during intrauterine life, α-tocopherol during lactation programmed increased blood pressure, hyporesponsiveness to AngII-induced hypertension and cardiac atrophy. The window for α-tocopherol programming cardiovascular alterations was extended along the post-weaning period. α-Tocopherol supplementation after weaning had a pro-oxidative effect in the left ventricle, which was likely responsible for AngII-induced tachycardia in rats prenatally exposed to high sodium.

Reduced cardiac weight index and reduced expression of β1-adrenoreceptor in the left ventricle of control and saline rats treated with α-tocopherol during lactation, seem to be consequential to impaired cardiac development. Along the first post-natal days, a transition of

cardiomyocyte proliferation to binucleation occurs, starting between post-natal day 3 and 4, and lasting up to postnatal day 12 (7,8,24). Presumably, in the present study cardiomyocyte proliferation was mainly affected, since cardiomyocyte hypertrophy, which spans throughout the post-weaning period, does not seem to be affected. Neither the cardiac weight index nor the β1-adrenoreceptor expression were reduced in the animals treated with α-tocopherol after weaning. Elevated gene expression for β1-adrenoreceptor in fetal heart and its lowered expression in neonatal heart points to the fact that β -adrenoreceptor is seminal to cardiac development (25). The β1-adrenoreceptor reduced expression in the left ventricle of the CTL and STL groups, points to delayed cardiac development. In line with present findings, an association of vitamins C and E was capable of reducing the left ventricle wall thickness in rats, when administered on post-natal days 1 to 6 (26). The anti-oxidant role of these substances might be a natural way to impair cardiac development by reducing angiogenesis (11).

Early reduction in cardiac ROS warranted by α-tocopherol, likely impaired cardiac development and changed irreversibly cardiac mass and beta-adrenoreceptor. The reduced inotropy that characterizes beta-adrenoreceptor deficiency could account for increased plasma renin activity and increased endogenous AngII (27). Increased endogenous AngII, represents a likely hemodynamic adjustment, accountable for increased tcSBP and MAP in the CTL group and increased MAP in the STL group. This assumption is based on the unresponsiveness to AngII-induced hypertension presented by groups CTL and STL. Hyporesponsiveness to AngII has been seen in conditions of hypovolemia (28-30), hypocalemia (31), hyperthermia (32) and hepatic cirrhosis (33). More recently, hypertension and hyporesponsiveness to AngII has been seen in caveolin-1 knockout rats and has been associated to an impaired uptake of AngII in the proximal tubule (34). Certainly, any of these reasons are applied to the present data. Albeit, urinary $K^+$ excretion was increased in some

moments when the animals were evaluated, we could not affirm hypocalemia or hypovolemia had occurred.

It is noteworthy to highlight that α-tocopherol treatment during lactation did not induce change in $O_2^-$ levels in the left ventricle, while it augmented $O_2^-$ levels when administered after weaning. A programmed pro-oxidative action of this vitamin, attributed to a supraphysiological dosage, could be argued if the same group (STW) showing high NADPH-dependent $O_2^-$ in the left ventricle (Fig. 7B) did not show reduction in this parameter in the kidneys (Table 1). Furthermore, this same dosage has shown to decrease hepatic oxidative stress in adult nursing rats (5). Again, an argument in favor of pro-oxidative action of α-tocopherol post-weaning treatment: the CTW group exhibited elevated basal and NADPH-induced $O_2^-$ levels (Fig. 7C). The α-tocopherol dosage used represents a supraphysiolgical content of this vitamin comparing to the α-tocopherol daily intake through commercial chow. Ordinarily, the standard diet warrants an average of 10 mg/100 g of BW. In addition, maize oil provides an insignificant amount, 0.01 mg of total tocopherols/100 g of BW daily. When administered to mothers during lactation, α-tocopherol is transferred through the milk (35) and some fraction of the antioxidant is distributed into the maternal body. On the other hand, when administered during the post-weaning period, it is distributed only throughout the tissue of the juvenile rats. A long-term vitamin E supplementation in normal pigs leads to increased levels of oxidative stress in the heart arterial wall, which may be partly related to an impairment of dimerization in endothelial nitric oxide synthase and/or a direct pro-oxidative effect of vitamin radicals (36).

The STW group showing the highest level of β1-adrenoreceptor (Fig. 5) and the highest difference between NADPH-oxidase stimulated $O_2^-$ levels and basal levels of $O_2^-$ (Fig. 7C), exhibited also compromised baroreflex sensitivity, once it showed increased heart rate during AngII-induced hypertension. In an effort to further clarify the repercussion of β1-

adrenoreceptor alterations, we evaluated the activity of plasma membrane $Ca^{2+}$-ATPase (PMCA) and sarcoplasmic reticulum $Ca^{2+}$-ATPase (SERCA). During cardiac relaxation, most of the intracellular calcium is transported back into the sarcoplasmic reticulum by SERCA, while a small proportion of calcium is ejected out of the cell by PMCA and sodium–calcium exchanger (37). PMCA plays a minor role in the bulk removal of intracellular calcium compared to the sodium–calcium exchanger (38). Instead, one isoform of PMCA, the PMCA 4, acts as a signaling molecule that regulates neuronal nitric oxide synthase, which is upstream to regulate β1-adrenoreceptor activity regulation (38). Surprisingly, these enzymes were unaltered in the groups treated at post-weaning, CTW and STW. Conversely, the group that shows consistently elevated tcSBP and MAP, the CTL group, also shows reduction in SERCA. It is early to bring forward the significance of this data. Nevertheless, diminished SERCA activity is correlated to reduced transient $Ca^{2+}$ and increased diastolic $Ca^{2+}$ in sarcolemma which could lead to diastolic dysfunction (39). Furthermore, SERCA activity is reduced in juvenile SHR immediately after the onset of hypertension (40).

Pre-natal saline treatment was evidenced by the higher body weight gain along development that has already been observed in previous reports (41, 42). The reason for high body weight could be attributed to metabolic dysfunction (43) or, more unlikely, to increased $Na^+$ accumulation (44). Pre-natal saline treatment was also evidenced by the reduced basal $O_2^-$ levels in the left ventricle (Fig. 7A, SV group) and by compromised baroreflex sensitivity (Fig. 4E, STW group).

The renal contribution to the elevated blood pressure in the CTL and STL groups must be considered, even if the renal functional parameters evaluated were unaltered. Except for the fact that α-tocopherol reduced the oxidative stress in the STW group, all the other functional parameters were similar among the groups. Previous results from our Laboratory

have shown that α-tocopherol augments AngII in the kidneys and changes its membrane, signaling to $Na^+$ transporters in the proximal tubule (6).

In summary, α-tocopherol during lactation impaired cardiac development and programmed elevation in blood pressure, independently of the nature of the fluid that the mothers received during pregnancy, water or NaCl solution. Whenever administered for a short period after weaning, α-tocopherol led to reduction in baroreflex sensitivity in rats that received sodium overload pre-natally. The treatment during lactation was not linked to changes in cardiac or renal oxidative stress. However, the post-weaning treatment was correlated to increased oxidative stress in the heart. These findings indicate that vitamin E overload during the early period of development, may be an etiological agent to later hypertension.

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**TABLE 1** Malondialdehyde (MDA), reduced glutathione (GSH), reactive oxygen species (ROS), and superoxide ($O_2^-$) production before and after adding NADPH in the kidney of rats at age of 210 days.

| | CV | CTL | CTW | SV | STL | STW |
|---|---|---|---|---|---|---|
| *MDA, nmol/mg protein* | 0.25 ± 0.02 | 0.22 ± 0.02 | 0.23 ± 0.02 | 0.24 ± 0.02 | 0.25 ± 0.02 | 0.22 ± 0.04 |
| GSH, *nmol/mg protein* | 104 ± 15 | 110 ± 12 | 112 ± 21 | 117 ± 3 | 97 ± 11 | 120 ± 14 |
| ROS, *IU/mg protein* | 4.33 ± 0.48 | 4 ± 0.63 | 3.65 ± 0.36 | 4.26 ± 0.61 | 3.50 ± 0.39 | 3.42 ± 0.60 |
| $O_2^-$, *$x10^3$RLU/mg protein* | | | | | | |
| Basal | 44± 10 | 67± 8 | 39 ± 5 | 46± 9 | 38± 7 | 42± 4 |
| NADPH-stimulated | 134± 13[a] | 116± 10[a] | 116± 11[a,b] | 162± 11[a] | 161± 22[a] | 111± 10[b] |

Values are mean ± SEMs, n = 7 to 12 rats. Means not sharing the same letters are different, $P < 0.05$. RLU, relative light units; IU, international unit; CV, rats prenatally provided with tap water and treated with α-tocopherol vehicle along lactation; CTL, rats prenatally provided with tap water and treated with α-tocopherol along lactation; CTW, rats prenatally provided with tap water and treated with α-tocopherol after lactation, for 3 weeks; SV, rats prenatally provided with 0.3 M NaCl solution and treated with α-tocopherol vehicle along lactation; STL, rats prenatally provided with 0.3 M NaCl solution and treated with α-tocopherol along lactation; STW rats prenatally provided with 0.3 M NaCl solution and treated with α-tocopherol after lactation, for 3 weeks.

**TABLE 2** Urinary potassium excretion ($U_K^+V$) and 24-h urinary protein ($U_{Prot}$) in rats at age of 150, 180 and 210 days.

| | CV | CTL | CTW | SV | STL | STW |
|---|---|---|---|---|---|---|
| $U_K^+V$, *mmol/100 g BW/24 h* | | | | | | |
| 150 days of age | $2.1 \pm 0.2^a$ | $2.0 \pm 0.1^a$ | $2.0 \pm 0.1^a$ | $1.4 \pm 0.30^b$ | $1.7 \pm 0.1^{a,b}$ | $1.5 \pm 0.1^{a,b}$ |
| 180 days of age | $2.1 \pm 0.1^a$ | $1.5 \pm 0.1^{b,c}$ | $1.7 \pm 0.1^{b,c}$ | $1.8 \pm 0.1^{a,b}$ | $1.4 \pm 0.1^{b,c}$ | $2.0 \pm 0.1^a$ |
| 210 days of age | $1.5 \pm 0.1$ | $1.5 \pm 0.1$ | $1.7 \pm 0.1$ | $1.4 \pm 0.1$ | $1.4 \pm 0.1$ | $1.5 \pm 0.0$ |
| $U_{Prot}$, *mg/100 g BW/24 h* | | | | | | |
| 150 days of age | $35 \pm 1^a$ | $36 \pm 1^a$ | $34 \pm 1^a$ | $30 \pm 2^a$ | $33 \pm 1^a$ | $31 \pm 2^a$ |
| 180 days of age | $41 \pm 1^a$ | $36 \pm 1^{a,b}$ | $35 \pm 1^b$ | $34 \pm 1^{b,c}$ | $32 \pm 2^c$ | $37 \pm 1^b$ |
| 210 days of age | $36 \pm 1$ | $35 \pm 1$ | $39 \pm 1$ | $38 \pm 2$ | $34 \pm 2$ | $35 \pm 1$ |

Values are mean ± SEMs, n = 7 to 12 rats. Means not sharing the same letters are different, $P < 0.05$. BW, body weight; CV, rats prenatally provided with tap water and treated with α-tocopherol vehicle along lactation; CTL, rats prenatally provided with tap water and treated with α-tocopherol along lactation; CTW, rats prenatally provided with tap water and treated with α-tocopherol after lactation, for 3 weeks; SV, rats prenatally provided with 0.3 M NaCl solution and treated with α-tocopherol vehicle along lactation; STL, rats prenatally provided with 0.3 M NaCl solution and treated with α-tocopherol along lactation; STW rats prenatally provided with 0.3 M NaCl solution and treated with α-tocopherol after lactation, for 3 weeks.

**Figure Legends**

**FIGURE 1** Body weight development of rats prenatally provided with tap water (A) or rats provided with 0.3 M saline solution (B). Illustration of the body weight difference between rats provided with tap water and saline solution (C). Values are mean ± SEMs, n = 7 to 12 rats. *$P < 0.05$: CTL vs. CV. CV, rats prenatally provided with tap water and treated with α-tocopherol vehicle along lactation; CTL, rats prenatally provided with tap water and treated with α-tocopherol along lactation; CTW, rats prenatally provided with tap water and treated with α-tocopherol after lactation, for 3 weeks; SV, rats prenatally provided with 0.3 M NaCl solution and treated with α-tocopherol vehicle along lactation; STL, rats prenatally provided with 0.3 M NaCl solution and treated with α-tocopherol along lactation; STW rats prenatally provided with 0.3 M NaCl solution and treated with α-tocopherol after lactation, for 3 weeks.

**FIGURE 2** The ratio heart weight/body weight (A) and kidney weight/body weight (B), at age of 210 days. Values are mean ± SEMs, n = 7 to 12 rats. Means not sharing the same letters are different, $P < 0.05$. Groups are described in Fig. 1.

**FIGURE 3** Tail-cuff systolic blood pressure (tcSBP) at ages of 150, 180 and 210 days. Values are mean ± SEMs, n = 7 to 12 rats. Means not sharing the same letters are different, $P < 0.05$. Groups are described in Fig. 1.

**FIGURE 4** Mean arterial pressure (MAP) evaluated at age of 210 days, before AngII infusion (A). AngII-induced MAP change (B). Heart rate (HR) registered during MAP measurement, before AngII infusion (C). AngII-induced HR change (D). The ratio AngII-induced HR change/ AngII-induced MAP change that means baroreflex (BRS) sensitivity (E). Values are

mean ± SEMs, n = 4 to 6 rats. Means not sharing the same letters are different, P < 0.05. Groups are described in Fig. 1.

**FIGURE 5** Representative immunodetection (A) and densitometric evaluation of β1-adrenoreceptor in the left ventricle of rats at age of 210 days. Values are mean ± SEMs, n = 4 to 5 samples. Means not sharing the same letters are different, P < 0.05. Groups are described in Fig. 1.

**FIGURE 6** Plasma membrane $Ca^{2+}$-ATPase (PMCA) and sarcoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) activity in the left ventricle of rats at age of 210 days (A). The ratioPMCA/SERCA (B). Values are mean ± SEMs, n = 4 to 6 samples. Means not sharing the same letters are different, P < 0.05. Groups are described in Fig. 1.

**FIGURE 7** Superoxide anions production in the left ventricle of rats at age of 210 days, expressed as relative light units (RLU), before (A) and after adding NADPH (B). Superoxide anions NADPH-oxidase dependent minus basal (C). Values are mean ± SEMs, n = 6 samples. Means not sharing the same letters are different, P < 0.05. Groups are described in Fig. 1.

**FIGURE 8** Urinary sodium excretion (UNa+V, in panel A). Creatinine clearance (B) in rats at age of 150, 180 and 210 days. Values are mean ± SEMs, n = 7 to 12 rats. Means not sharing the same letters are different, P < 0.05. Groups are described in Fig. 1.

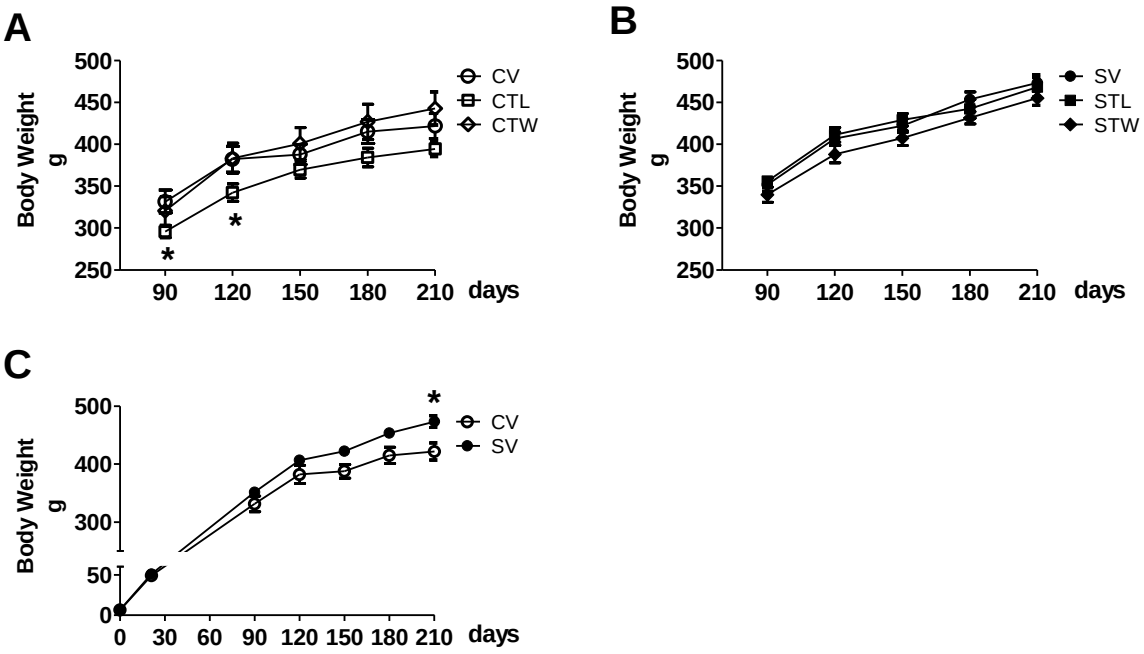
A
Body Weight
g
500
450
400
350
300
250
90
120
150
180
210
days
CV
CTL
CTW
*
*
B
Body Weight
g
500
450
400
350
300
250
90
120
150
180
210
days
SV
STL
STW
C
Body Weight
g
500
400
300
50
0
0
30
60
90
120
150
180
210
days
CV
SV
*

**FIGURE 1**

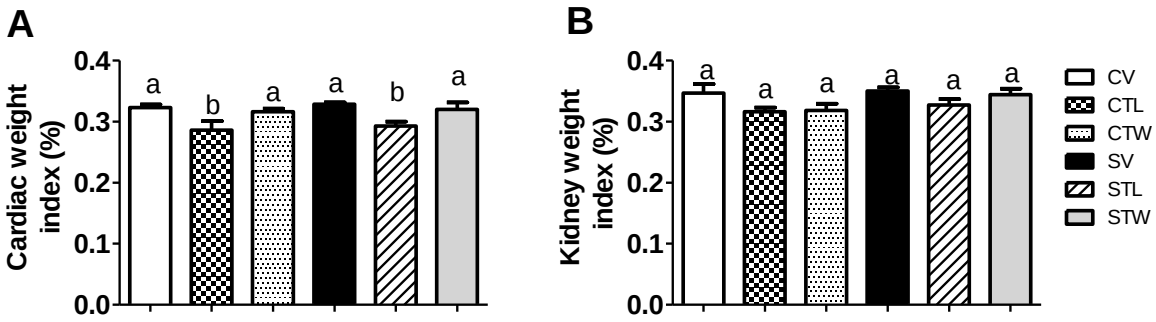
A
Cardiac weight index (%)
0.4
0.3
0.2
0.1
0.0
a
b
a
a
b
a
B
Kidney weight index (%)
0.4
0.3
0.2
0.1
0.0
a
a
a
a
a
a
CV
CTL
CTW
SV
STL
STW

**FIGURE 2**

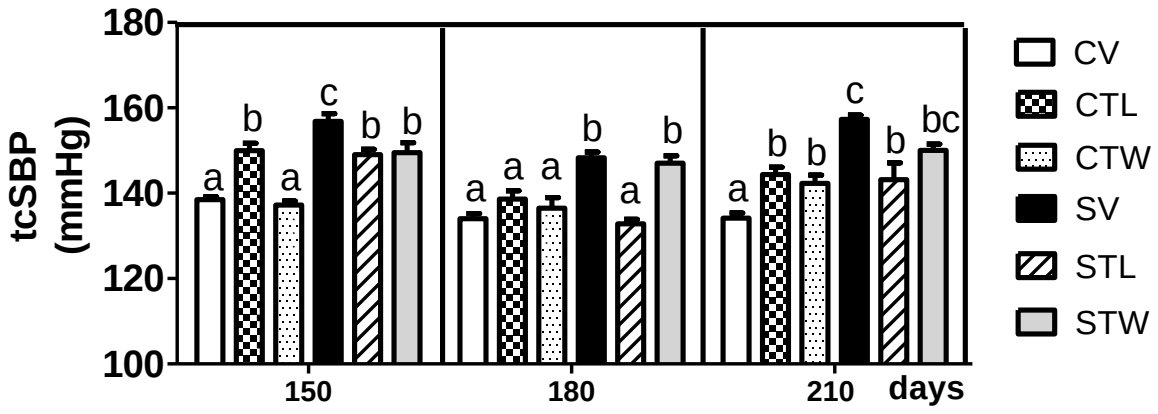
180
160
140
120
100
tcSBP
(mmHg)
a
b
a
c
b
b
a
a
a
b
a
b
a
b
b
c
b
bc
150
180
210
days
CV
CTL
CTW
SV
STL
STW

**FIGURE 3**

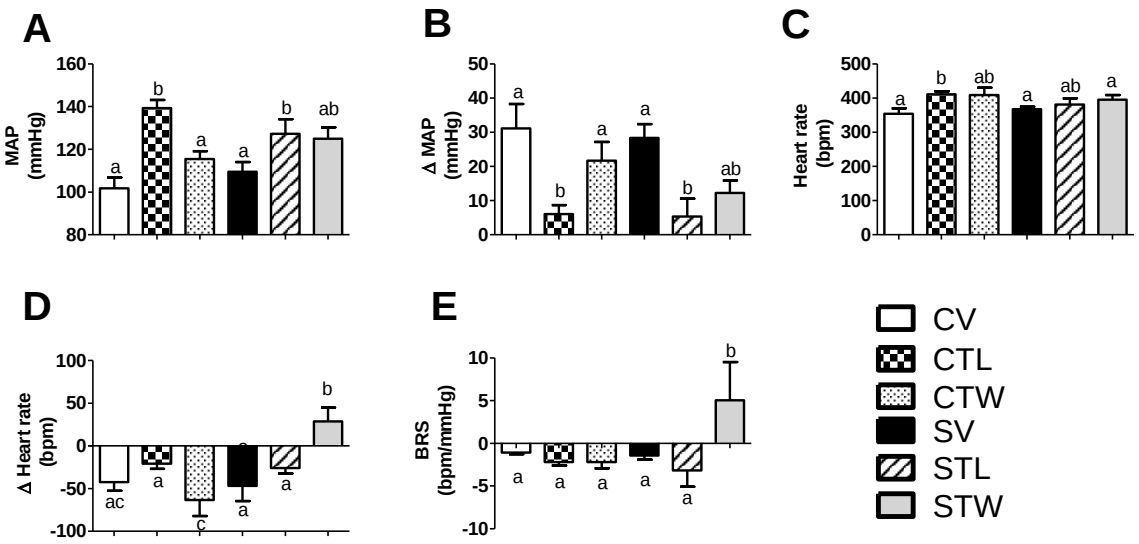
A
MAP (mmHg)
160
140
120
100
80
a
b
a
a
b
ab
B
Δ MAP (mmHg)
50
40
30
20
10
0
a
b
a
a
b
ab
C
Heart rate (bpm)
500
400
300
200
100
0
a
b
ab
a
ab
a
D
Δ Heart rate (bpm)
100
50
0
-50
-100
ac
a
c
a
a
b
E
BRS (bpm/mmHg)
10
5
0
-5
-10
a
a
a
a
a
b
CV
CTL
CTW
SV
STL
STW

**FIGURE 4**

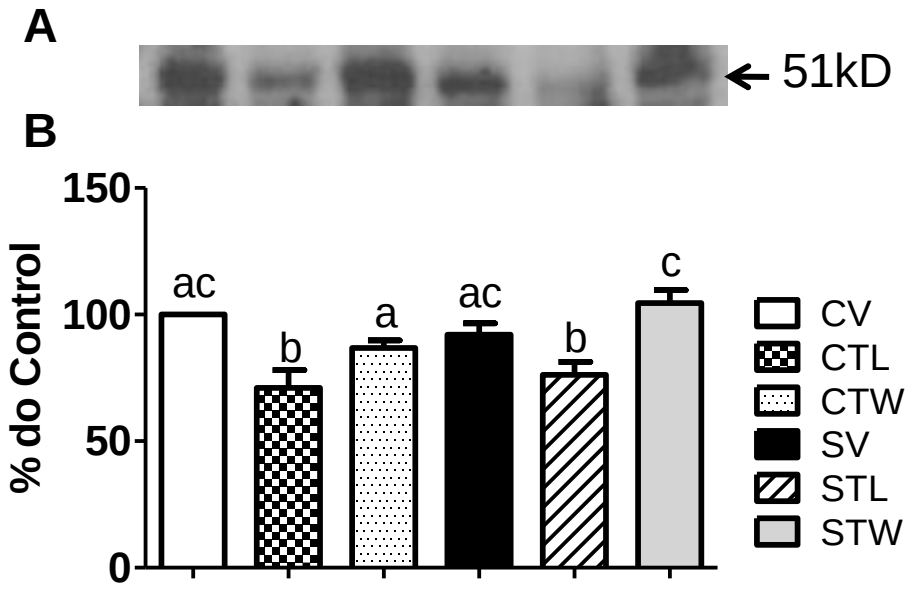


**FIGURE 5**

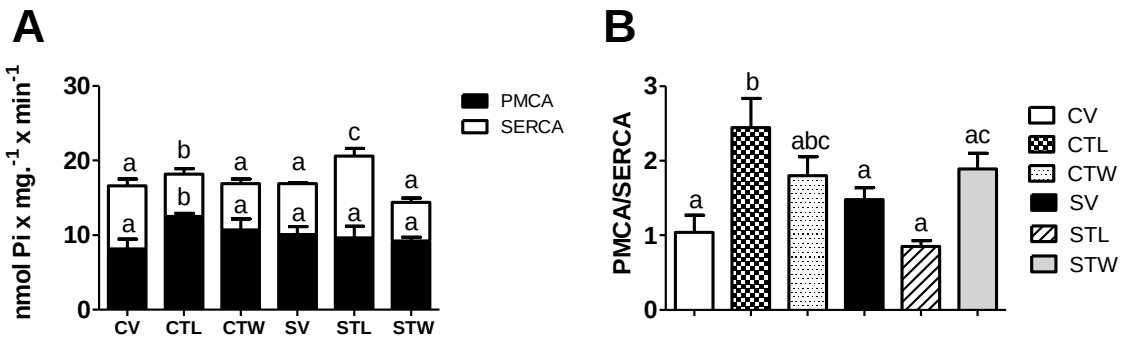
A
nmol Pi x mg.-1 x min-1
30
20
10
0
a
a
b
b
a
a
a
a
c
a
a
a
CV
CTL
CTW
SV
STL
STW
PMCA
SERCA
B
PMCA/SERCA
3
2
1
0
a
b
abc
a
a
ac
CV
CTL
CTW
SV
STL
STW

**FIGURE 6**

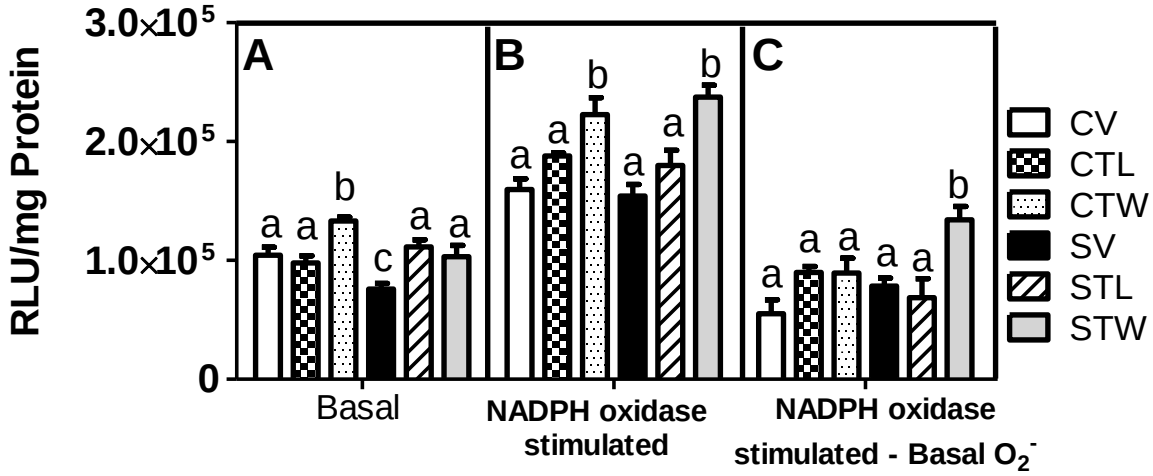
RLU/mg Protein
3.0×10⁵
2.0×10⁵
1.0×10⁵
0
A
B
C
a a b c a a
a a b a a b
a a a a a b
CV
CTL
CTW
SV
STL
STW
Basal
NADPH oxidase stimulated
NADPH oxidase stimulated - Basal O2-

**FIGURE 7**

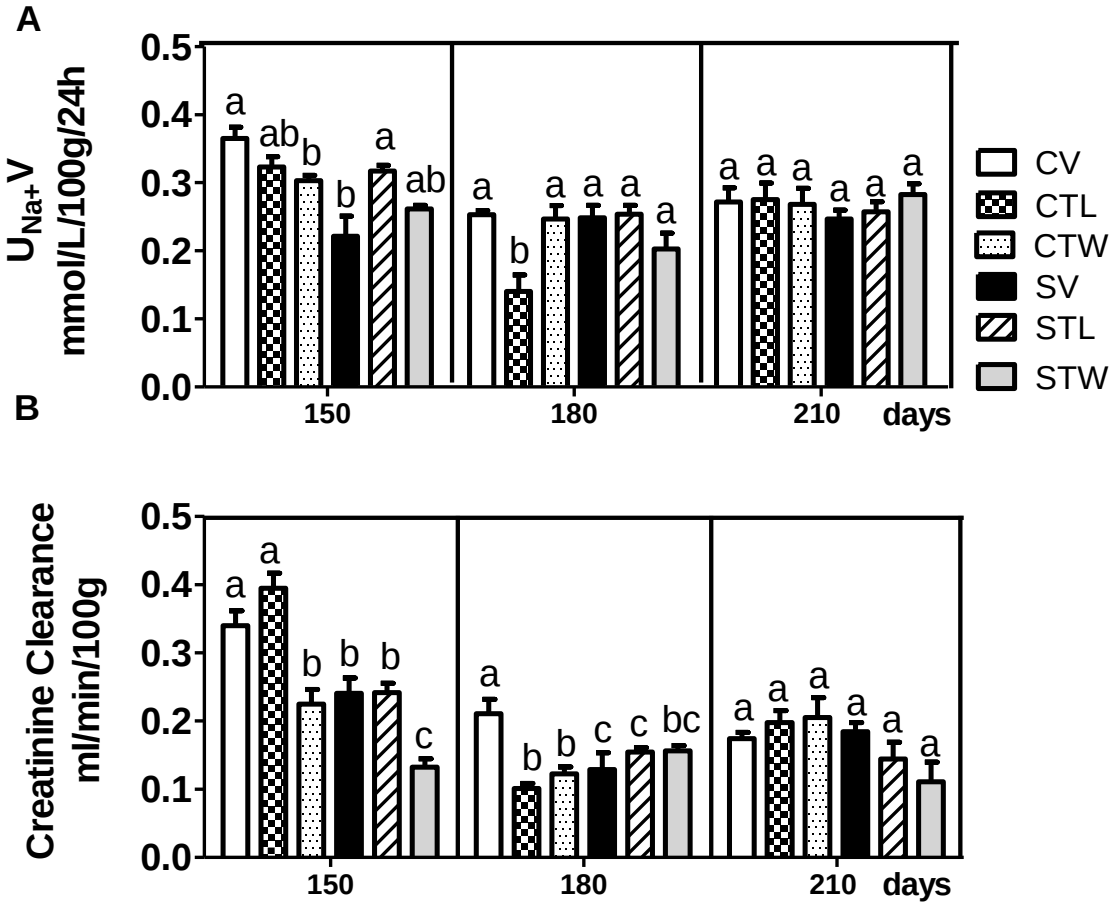
A
$U_{Na+}V$
mmol/L/100g/24h
0.5
0.4
0.3
0.2
0.1
0.0
a ab b b a ab
a b a a a a
a a a a a a
150
180
210
days
CV
CTL
CTW
SV
STL
STW
B
Creatinine Clearance
ml/min/100g
0.5
0.4
0.3
0.2
0.1
0.0
a a b b b c
a b b c c bc
a a a a a a
150
180
210
days

**FIGURE 8**

# ANEXO III

**Perinatal sodium overload triggers hyporresponsiveness to angiotensin II-induced hypertension: Enalapril shortly after weaning recover it.**

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**Abstract**

It is known that a moderate sodium overload, 0.17 M, given as drinking water during pregnancy and weaning, is capable to change some parameters of offspring renal function, but it is not capable to change blood pressure. In an attempt to clarify hypertension pathophysiology, in the present work, it was investigated whether a higher sodium overload was capable to induce alterations in renal oxidative stress and consequential hypertension in the offspring. To investigate the role of angiotensin II (AngII) in the programmed alterations, the offspring received enalapril after weaning, for 3 weeks. Female Wistar rats received tap water along all study to compose the control group (n=3), while some rats received 0.3 M NaCl solution from twenty days before pregnancy up to weaning, the saline group (n=3). Tail-cuff systolic blood pressure was measured from the age of 25 days until 145 days. 24-h urinary protein and glomerular filtration rate were evaluated. Mean arterial pressure was measured in anesthetized rats before and during AngII infusion (100 ng/kg/min, iv). Lipid peroxidation was measured by the levels of malondialdehyde (MDA), as well as the levels of reduced glutathione (GSH) was measured in the kidney and liver. Perinatal treatment with saline did not change MDA levels in the kidney, but enalapril increased it in the kidney of control rats. None of these treatments changed the 24-h urinary protein nor the glomerular filtration rate. Neither the perinatal saline treatment nor the enalapril changed the blood pressure at adult life. However, the rats perinatally treated with sodium overload showed hyporesponsiveness to AngII-induced hypertension. The treatment with enalapril recovered the responsiveness to AngII. However, enalapril provoked cardiac hypertrophy in control rats and exacerbated saline-induced cardiac hypertrophy.

## Introduction

Offspring born from mothers maintained on a diet containing 3% of sodium, which is high compared to 0.51% considered an intermediate content, along pregnancy and lactation shows lowered number of nephrons (1). To rats, dietary NaCl intake is considered normal up to 1.1% (2). On the other hand, perinatal sodium overload throughout a 0.17 M NaCl solution changes renal function at adult life, in part by increasing oxidative stress (3), and in part by changing the subcellular pathway between angiotensin II (AngII) and the $Na^+$ATPase transporter in the proximal tubule (4), but it did not change the number of nephrons.

Ordinarily, control dams intake an average of 100 mg of sodium per day, considering the content of sodium standard diet. Drinking a 0.17 M NaCl solution confers a daily intake of sodium in average of 700 mg. This means an important overload that could not be applied in terms of human sodium intake, but represents a tool to understand the role of sodium overload on organogenesis. Dams on a 0.3 M NaCl solution, in the drinking water, from pregnancy day 12 to 19 show hypertension that is not manifested in nonpregnant rats (5).

On the other hand, it is known that short treatments with angiotensin converting enzyme inhibitor (ACEI) are sufficient to change the course of hypertension in rats (6,7). Furthermore, there is evidence that enalapril, one ACEI, is capable to reduce the $(Na^+ + K^+)$ATPase and $Na^+$-ATPase activities in the proximal tubule of adult rats (3), when it is administered after weaning, for 3 weeks.

In the present work, it was investigated whether a perinatal maternal sodium overload higher than that one previously employed (3,4) is able to induce additional alterations in oxidative stress and consequential hypertension. To investigate the role of angiotensin II

(AngII) in the programmed alterations, enalapril, administered after weaning for 3 weeks, was associated to the perinatal treatment.

## Material and Methods

### *Ethical Considerations*

Experimental protocol followed the guidelines and had approval of the Committee for Experimental and Animal Ethics at the Federal University of Pernambuco (no. 23076.055063/2010-03).

### *Animals*

Wistar rats were used throughout the study. Rats were maintained in a 12:12 h light/dark cycle at 23 ± 2°C and 55 ± 5% humidity. Female rats, 70 days old, weighing 170 to 200 g, were randomly allocated to receive tap water *ad libitum*, the control dams (C, n = 3), or 0.3 M saline, the saline group (S, n = 3), until the weaning. All rats were maintained on a standard diet (Purina Agribands, Paulınia, SP, Brazil) *ad libitum*. They were mated at 90 days and the first pregnancy day was detected by the presence of a vaginal plug. Male offspring was treated with enalapril (E, 100 mg/l) from weaning, at age of 21 days, for 3 weeks. Thus, four groups were studied: control and saline offspring maintained on tap water, the groups C (n = 5) and S (n = 5), respectively; and control and saline offspring treated with enalapril, the groups CE (n = 6) and SE (n = 5). The enalapril regimen resulted in an average of 4 mg per rat per day during the three weeks after weaning.

### *Blood pressure and parameters of cardiac and renal function*

Tail-cuff systolic blood pressure (tcSBP) was measured by plethysmography (IITC Life Science B60-7/16'', Life Science Instruments, Woodland Wills, CA) along the enalapril

treatment until the age of 145 days. To measure tcSBP, animals were acclimated to the room, and to cages of containment. Acclimation was performed for three days, when the room was kept silent and maintained under temperature around 22°C. To measure tcSBP the animals were warmed at 36.6°C. tcSBP was obtained as a mean from an average of 3 to 5 measurements, per rat, at each experiment. Creatinine clearance, 24-h proteinuria ($U_{Prot}$) and urinary $Na^+$ and $K^+$ excretion were also evaluated until age of 210 days. Metabolic cages (Tecniplast Gazzada Sarl, Buguggiate, Italy) were used to collect 24-h urine samples for measuring proteinuria, creatinine and $Na^+$. Blood samples were obtained from the caudal artery for creatinine measurements. At age of 210 days, 48 h after completion of the metabolic studies, animals were anesthetized using pentobarbital (60 mg/kg, ip) and surgically prepared for direct measurement of mean arterial pressure (MAP) and heart rate (HR) before and after AngII infusion (100 ng/kg/min, iv). Femoral artery was catheterized and connected to a blood pressure transducer (SP 844, MEMSCAP Inc, Durham, USA) assembled to a data acquisition unity (Power Lab, 8/30, ML866, ADInstruments, Australia), while the right jugular vein was catheterized to AngII infusion (11 plus, Harvard Apparatus, Holliston, MA, USA). MAP and HR were continuously registered for 20 min, by using a Lab Chart software (7.3.4 version, ADInstruments). Yet under anesthesia, the hearts and kidneys were withdrawn, rapidly weighed and flash frozen in liquid nitrogen before to keep at – 80°C. The rats were euthanized by exsanguination.

*Malondialdehyde (MDA) and reduced glutathione (GSH) in kidney and liver*

Tissues were homogenized in 150 mM KCl (1g tissue:5 ml ratio). The levels of MDA were measured according previously with some modifications. For the standard curve, 1,1,3,3-tetraethoxy-propane was used. Levels of GSH were assessed as non-protein

sulfhydryl groups. L-cysteine was used for the standard curve. Both the MDA and GSH results were corrected for protein concentration. The assays were performed in duplicates.

*Analytical methods.* Protein in tissue samples and urinary protein was measured using the Folin phenol method. Urinary $Na^+$ and $K^+$ were measured using an electrolyte analyzer (AVL 9180, Roche Diagnostics GmbH, Mannheim, Germany). Serum creatinine was determined using a commercial kit.

*Statistical Analyses.* The results are expressed as mean ± SEMs. Differences between experimental groups were analyzed using two-way ANOVA followed by Bonferroni test, while difference between Ang II-stimulated MAP and HR versus basal conditions were analyzed by paired Student's *t* test.

**Results**

*Body weight and organ index*

Figure 1 shows the body weight development that was similar among the four groups. However, S, CE and SE groups showed increased cardiac weight index compared to C group. Further, cardiac index was higher in SE than in S group (Figure 2A). Kidney weight index was increased in S group compared to C group (Figure 2B).

*Blood Pressure and Heart rate*

Figure 3 shows that tcSBP was lowered in CE and SE groups at age of 25 days, when enalapril treatment was in course. Except for this anti-hypertensive effect of enalapril, tcSBP was similar among the groups. Basal MAP was also similar among the groups (Figure 4A). However, the increment in MAP in response to AngII infusion was lower in S than in C group

and also lower in CE and SE than in their respective groups Figure 4B). Basal HR was similar among the four groups (Figure 4C) and changed similarly among the groups in response to AngII (Figure 4D).

*Malondialdehyde and reduced glutathione in kidney and liver*

MDA was higher in the kidney of CE group compared to C group and lowered in the liver of S group compared to C group (Figure 5A). GSH in the kidney was similar among the groups (Figure 5B), but it was lowered in the liver of S and CE groups compared to C group (Figure 5B).

**Conclusions**

Increasing maternal sodium overload from 0.17 M (3,4) to 0.3 M, throughout the drinking water, from twenty days before pregnancy up to the weaning, did not increase oxidative stress in the offspring kidney nor led to increased blood pressure. However, this maternal treatment led to hyporresponsiveness to angiotensin II-induced hypertension. Enalapril treatment over three weeks after the weaning recover the responsiveness to angiotensin II. Nonetheless, enalapril treatment provoked cardiac hypertrophy.

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**Figure Captions**

Figure 1: Body weight development. C and S are, respectively, control and saline offspring maintained on tap water and saline. CE and SE are, respectively control and saline offspring treated with enalapril. Results are mean ± SEM.

Figure 2: Cardiac and renal weight index. See group descriptions in Figure 1. Results are mean ± SEM. Bars not sharing the same letters are different. $P<0.05$, two-way ANOVA followed by Bonferroni test.

Figure 3: Tail-cuff systolic blood pressure. See group descriptions in Figure 1. Results are mean ± SEM. Bars not sharing the same letters are different. $P<0.05$, two-way ANOVA followed by Bonferroni test.

Figure 4: Mean arterial pressure and heart rate. See group descriptions in Figure 1. (A) Mean arterial pressure (MAP). (B) AngII-induced change in MAP. (C) Heart rate. (D) AngII-induced change in heart rate. Results are mean ± SEM. Bars not sharing the same letters are different. $P<0.05$, two-way ANOVA followed by Bonferroni test (A and C), paired Student *t* test (B and D).

Figure 5: Malondialdehyde (MDA) and reduced glutathione (GSH) in kidney and liver. See group descriptions in Figure 1. Results are mean ± SEM. Bars not sharing the same letters are different. $P<0.05$, two-way ANOVA followed by Bonferroni test.

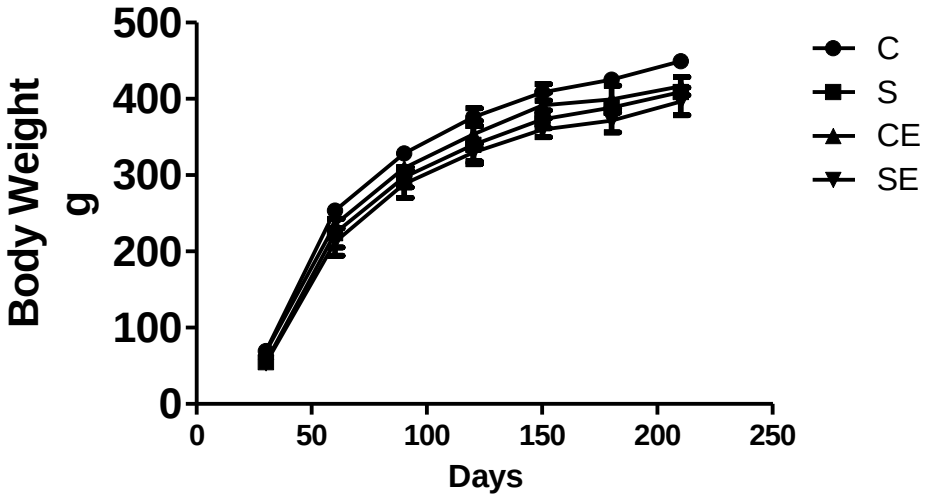
Body Weight
g
500
400
300
200
100
0
0
50
100
150
200
250
Days
C
S
CE
SE

**FIGURE 1**

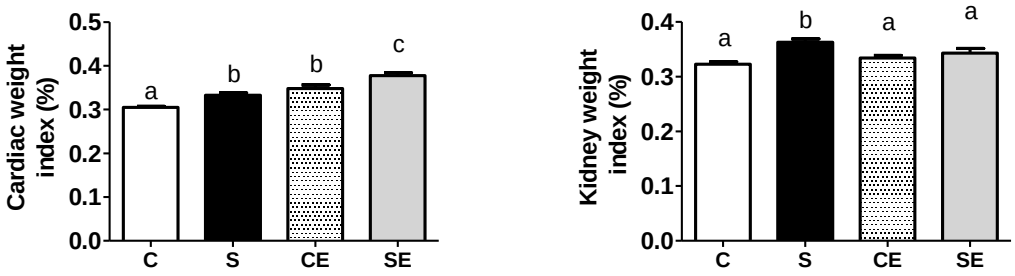
Cardiac weight index (%)
0.5
0.4
0.3
0.2
0.1
0.0
a
b
b
c
C
S
CE
SE
Kidney weight index (%)
0.4
0.3
0.2
0.1
0.0
a
b
a
a
C
S
CE
SE

**FIGURE 2**

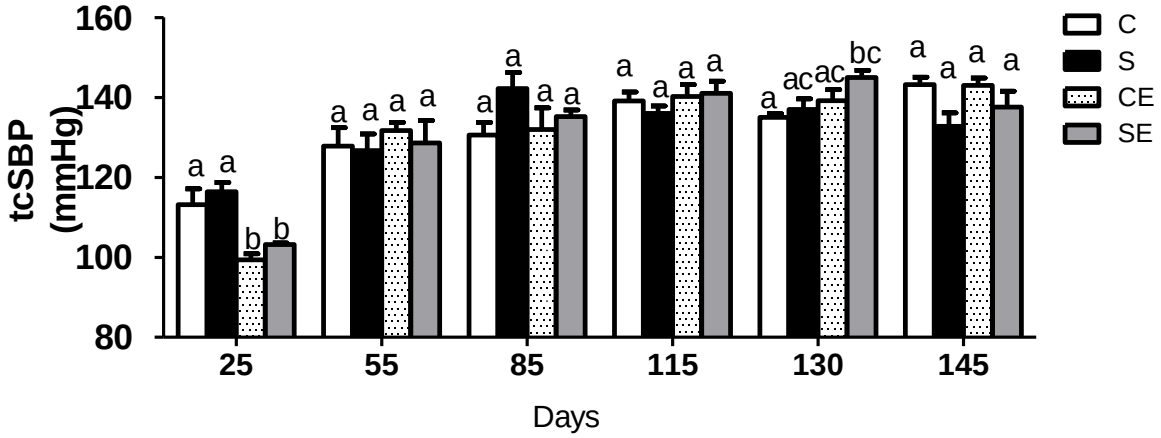


**FIGURE 3**

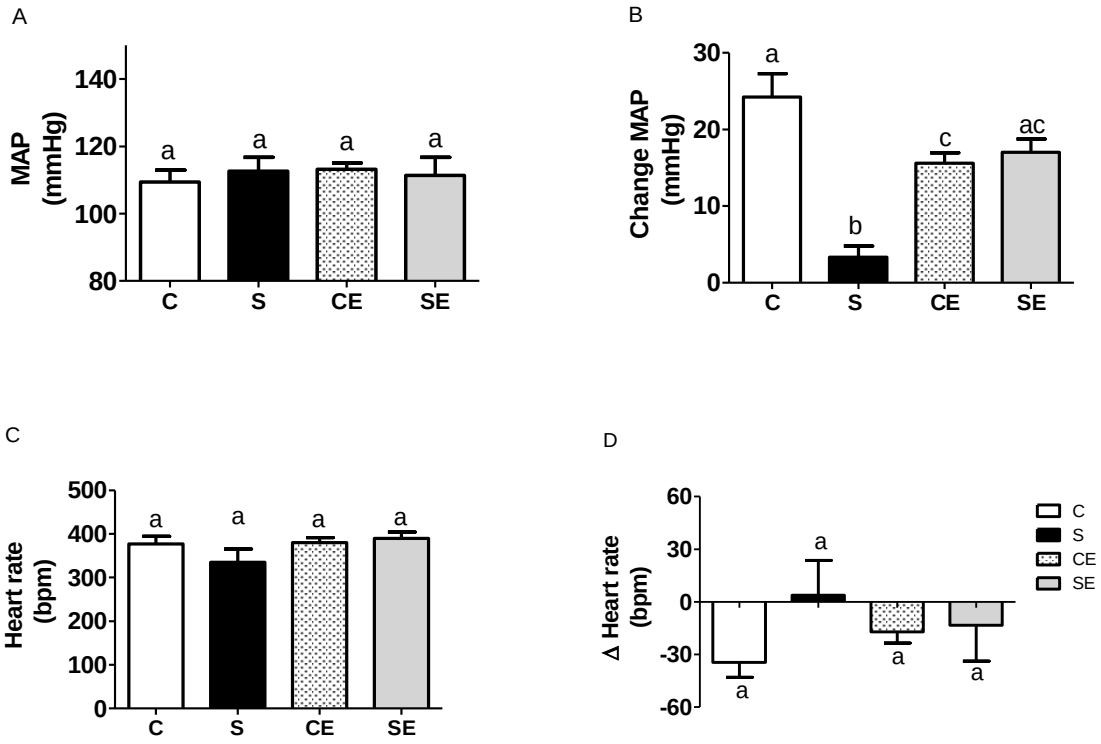
A
MAP (mmHg)
140
120
100
80
a
a
a
a
C
S
CE
SE
B
Change MAP (mmHg)
30
20
10
0
a
b
c
ac
C
S
CE
SE
C
Heart rate (bpm)
500
400
300
200
100
0
a
a
a
a
C
S
CE
SE
D
Δ Heart rate (bpm)
60
30
0
-30
-60
a
a
a
a
C
S
CE
SE

**FIGURE 4**

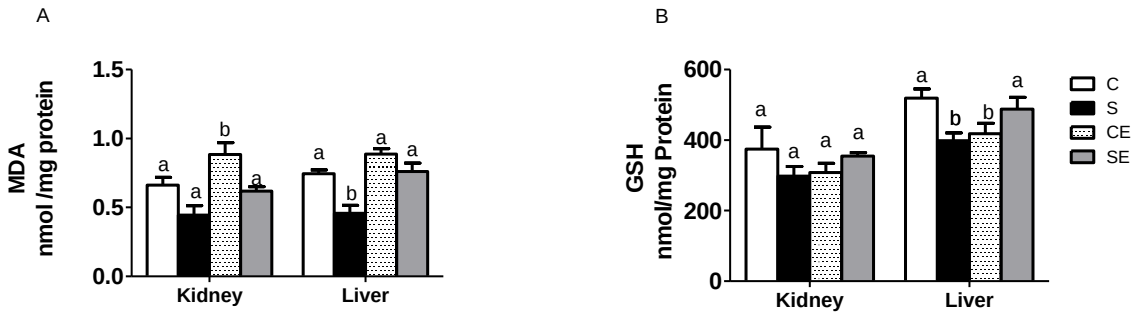
A
MDA
nmol /mg protein
1.5
1.0
0.5
0.0
a
a
b
a
a
b
a
a
Kidney
Liver
B
GSH
nmol/mg Protein
600
400
200
0
a
a
a
a
a
b
b
a
Kidney
Liver
C
S
CE
SE

**FIGURE 5**

# ANEXO IV

**Universidade Federal de Pernambuco**
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Recife, 15 de fevereiro de 2011.

Ofício nº 351/11

Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Profª: Ana Durce Oliveira da Paixão**
Departamento: Fisiologia e Farmacologia - UFPE
Processo nº 23076.055063/2010-03

Os membros da Comissão de Ética no Uso de Animais do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEUA-UFPE) avaliaram seu projeto de pesquisa intitulado, ***"Sobrecarga de sódio materna em ratos: Repercussão sobre a função renal da prole e possibilidades de reversão. "***

Concluímos que os procedimentos descritos para a utilização experimental dos animais encontram-se de acordo com as normas sugeridas pelo Colégio Brasileiro para Experimentação Animal e com as normas internacionais estabelecidas pelo National Institute of Health Guide for Care and Use of Laboratory Animals as quais são adotadas como critérios de avaliação e julgamento pela CEUA-UFPE.

Encontra-se de acordo com as normas vigentes no Brasil, especialmente a Lei 11.794 de 08 de outubro de 2008, que trata da questão do uso de animais para fins científicos e didáticos.

Diante do exposto, emitimos **parecer favorável** aos protocolos experimentais a serem realizados.

Observação: Origem dos animais: Biotério Setorial do Departamento de Fisiologia e Farmacologia; Animais: Ratos; Sexo: Machos e fêmeas; Linhagem: Wistar; Idade: 90 dias; Nº de Animais previsto no projeto: 72 animais.

Atenciosamente,

Profa. Maria Teresa Jansen
Presidente do CEUA
UFPE