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SOBRECARGA DE Na^+ DURANTE O PERÍODO PERINATAL PROGRAMA O AUMENTO DA REABSORÇÃO PROXIMAL DESTE ELETRÓLITO E HIPERTENSÃO EM RATOS ADULTOS: O TRATAMENTO COM ENALAPRIL DEPOIS DO DESMAME REPROGRAMA ESTAS ALTERAÇÕES

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“*O conhecimento nunca está terminado. É uma teia que vamos tecendo a partir da superação dos limites*.”

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SUMÁRIO

RESUMO

Foram investigados os mecanismos renais que permeiam a hipertensão induzida pela sobrecarga perinatal de Na^+, bem como, os efeitos de um tratamento de curta duração com o enalapril, em termos de reprogramação de alterações moleculares no rim e dos mecanismos responsáveis pela hipertensão. Ratos machos adultos Wistar foram obtidos de mães mantidas, durante a gravidez e lactação, com dieta padrão e água potável, o grupo controle, ou com NaCl 0,17 M, o grupo salina. O enalapril (100 mg/l) foi administrado durante três semanas, após o desmame. A prole de mães que beberam salina apresentou túbulos proximais com atividade aumentada da $(Na^+ + K^+)$ATPase sem alterações na expressão da subunidade alfa. A atividade da Na^+-ATPase insensível a ouabaina permaneceu inalterada, mas sua resposta à angiotensina II (ANG II) foi perdida. A atividade das proteína kinase C (PKC), da proteína kinase dependente de AMPc (PKA) e as substâncias reativas ao ácido tiobarbitúrico (TBARS) apresentaram-se aumentadas, enquanto a expressão de receptores AT_2 apresentou-se diminuída e a expressão de receptores AT_1 apresentou-se inalterada. O tratamento com enalapril reduziu as atividades de ambas bombas e as atividades da PKC e PKA na prole que foi exposta ao sódio durante o período perinatal. Adicionalmente, o tratamento com enalapril reduziu a expressão de receptores AT_2 e aumentou os níveis de TBARS no rim. A atividade reduzida da PKA na prole tratada com salina mais enalapril foi acompanhada por recuperação da atividade estimulatória, mas não da inibitória, no que diz respeito a ação da ANG II sobre a enzima Na^+-ATPase. A prole de mães tratadas com salina apresentou, aos 90 dias, pressão arterial mais elevada do que a prole de mães controle. O tratamento com enalapril preveniu a hipertensão. No entanto, os efeitos paralelos, como a diminuição da atividade da PKA e da PKC, assim como dos receptores AT_2 e o aumento de TBARS podem prejudicar a função renal na idade adulta.

Palavras-chave: Sobrecarga de sódio. $(Na^+ + K^+)$ATPase renal. Hipertensão. Programação renal. enalapril.

ABSTRACT

The renal mechanisms underlying perinatal Na^+ overload-induced hypertension were investigated, as were the effects of short treatment with enalapril in terms of the reprogramming of molecular alterations in the kidney and the onset of hypertension. 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) 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 activity without alterations in α-subunit expression. Ouabain-insensitive Na^+-ATPase activity remained unchanged but its response to angiotensin II (ANG II) was lost. Protein kinase C (PKC), cAMP-dependent protein kinase (PKA) and renal thiobarbituric acid reactive substances (TBARS) increased, AT_2 receptor expression decreased and AT_1 receptor expression remained unchanged. Early treatment with enalapril reduced the activities of both pumps, and reduced PKC and PKA activities depending on whether the offspring were exposed to high Na^+ perinatally. In addition, treatment with enalapril lowered AT_2 receptor expression and increased local TBARS. Reduced PKA activity in enalapril-treated saline offspring was accompanied by a recovery of the stimulatory, but not the inhibitory, response to ANG II. The ninety day old saline offspring had higher blood pressure than controls and early enalapril administration blocked the onset of hypertension, indicating reprogramming of $(Na^+ + K^+)$ATPase. Enalapril was beneficial in decreasing Na^+ reabsorption and preventing hypertension in adult offspring. However, side effects including down-regulation of PKA, PKC and AT_2 receptors, and increasing TBARS could impair renal function in later life.

Keywords: Na^+ overload; renal $(Na^+ + K^+)$ATPase; hypertension; renal programming; enalapril

1. INTRODUÇÃO

Nossos ancestrais alimentavam-se de uma dieta pobre em sódio, um hábito alimentar que foi mantido por algumas tribos indígenas, como os Yanomami, por exemplo, os quais apresentam níveis pressóricos cerca de 20 mmHg menores do que a média da população (MANCILHA-CARVALHO *et al*., 1989). No entanto, os hábitos alimentares ao longo dos anos têm passado por modificações que levaram adaptações orgânicas, especialmente dos rins (HALPERIN *et al*., 2006). Atualmente, nossos hábitos alimentares incluem altos teores de sódio, o que tem sido correlacionado com o aumento de doenças cardiovasculares e renais (GLEIBERMANN, 1973; MIMRAN *et al*., 2008). No Japão, por exemplo, ocorreu entre a década de 1950-1960 uma elevada incidência de acidente vascular cerebral e hipertensão que foi associada com alta ingestão de sódio na dieta (INSULL *et al*., 1968; SASAKI 1962, 1964). A elevada ingestão de sódio na dieta pode ser classificada como nutrição inadequada, ou seja, má-nutrição e como tal tem repercutido, como mencionado acima, quando vigente, sobre a elevada incidência de doenças cardiovasculares e renais. Adicionalmente a sobrecarga de sódio na dieta durante o período perinatal pode programar na prole alterações tardias e nas mães as doenças já mencionadas.

2. FUNDAMENTAÇÃO TEÓRICA

2.1 Sobrecarga de sódio durante a gestação e suas repercussões na saúde materna

A ingestão elevada de sódio durante a gravidez induz elevação da pressão arterial (BEAUSÉJOUR *et al*., 2003) e redução da pressão de perfusão uterina (LEE *et al.,* 1983, LEFFLER *et al*., 1986), tendo sido inclusive um modelo proposto de pré-eclampsia experimental (BEAUSÉJOUR *et al*., 2007). A hipertensão ocorre em parte, por alterações no balanço hidroeletrolítico devido à elevação da concentração de sódio plasmática e dos níveis de hematócrito associados com balanço hídrico negativo (BEAUSÉJOUR *et al*., 2003). Ao que concerne ao rim a alta ingestão de sódio materna eleva os níveis de estresse oxidativo renal (BEAUSÉJOUR *et al,*. 2007) e proteinúria (BEAUSÉJOUR *et al*., 2003; CARDOSO *et al*., 2009). A

depender do teor de sódio na dieta pode ocorrer alterações no ganho de peso materno. Beauséjour et al., (2003) utilizaram uma sobrecarga de 1,9 g de sódio/dia e observaram que o ganho de peso materno apresenta-se diminuído. Balbi et al., (2004) observaram que ratas submetidas a uma sobrecarga de 1 g de sódio/dia tiveram um ganho de peso mais elevado quando comparadas ao grupo controle. Cardoso et al., (2009) utilizando uma sobrecarga de 1,1 g de sódio/dia o ganho de peso materno não foi influenciado. Portanto, essas divergências sobre a alta ingestão de sódio materna e o ganho de peso durante a gestação pode estar relacionado ao teor de sódio utilizado e ao protocolo experimental, pois o inicio da administração do alto teor de sódio são diferentes nos três estudos acima citados.

É sabido que durante a gestação ocorre vasodilatação (NI *et al*., 1997, ST-LOUIS *et al*., 1997, 2001, ZWART *et al*., 1998). e ativação do sistema renina angiotensina aldosterona (SRAA) (SULLIVAN *et al*., 2004). O SRAA é modulado através do teor de sódio na dieta. Em ratas mantidas com uma dieta com elevado teor de sódio há uma diminuição na atividade da renina plasmática e dos níveis plasmáticos de aldosterona, o que sugere inibição do SRAA (BEAUSÉJOUR *et al*. 2003). Em relação à placenta a sobrecarga de sódio durante a gestação altera a expressão de RNAm para receptores de angiotensina II (ANG II). Quando uma sobrecarga de 2,88 g de sódio/dia é administrada ocorre um aumento na expressão de RNAm para receptores AT_1 e diminuição nós níveis teciduais placentários de angiotensina I (ANG I) e ANG II (LEANDRO *et al*., 2008). No entanto com uma sobrecarga de 1,9 g de sódio/dia foi correlacionada com a diminuição da expressão de RNAm para receptores AT_1 (BEAUSÉJOUR *et al*., 2003). Além de influenciar o SRAA na mãe, a sobrecarga materna de sódio pode induzir aumento do estresse oxidativo plancentário, levando a vasoconstrição placentária e comprometimento da nutrição fetal (BEAUSÉJOUR *et al*., 2007)

Dados do nosso laboratório demonstraram que a sobrecarga de sódio de 1,1 g de sódio/dia 20 dias antes da concepção não elevou os níveis de estresse oxidativo placentário (CARDOSO *et al*., 2009). No entanto, alta ingestão de sódio correspondente a 1,9 g de sódio/dia durante o 15-22º dias de gestação diminui o peso placentário e compromete o desenvolvimento fetal (BEAUSÉJOUR *et al*., 2003). Embora a alta ingestão de sódio materna utilizada em nosso trabalho não tenha elevado o estresse oxidativo placentário (CARDOSO *et al*., 2009), o sódio é transferida para o feto através do fluido amniótico (HAZON *et al*., 1998) e durante a

lactação é transferida para o lactente no leite materno (VINJANDE *et al*., 1996). Em humanos, tem-se demonstrado que a ingestão de leite materno pelo lactente é inversamente proporcional ao teor de sódio. Quanto menor a quantidade deste eletrólito no leite materno maior é a ingestão pelo lactente e vice e versa (MANGANARO *et al*., 2007).

2.2 Repercussões da sobrecarga de sódio perinatal sobre a função renal da prole

Ratos neonatos submetidos a sobrecarga pré-natal de sódio apresentam alterações nos elementos da formação estrutural renal. Há uma menor expressão de α-actina, fibronectina, antígeno nuclear de proliferação celular (PCNA), e ANG II no córtex renal nas regiões túbulo intersticial como glomerular (BALBI *et al*., 2004). Neste mesmo estudo se demonstra uma menor densidade de receptores AT_1 na região cortical renal.

Alta ingestão materna de sódio durante a gestação e lactação determina na prole maior apetite por sódio, durante a vida adulta (BIRD *et al*., 1987). Também tem sido evidenciado que a prole aos trinta dias de vida pós natal apresenta hipertensão (BALBI *et al*., 2004) bem como na idade adulta (NICOLANTONIO *et al*., 1987; Di NICOLANTONIO *et al*., 1990; CONTRERAS ,1993; HANZON *et al*., 1998; SILVA *et al*., 2003). Outra evidencia é que ratos machos adultos submetidos à sobrecarga de sódio perinatal apresenta uma sensibilidade ao sódio diminuído e a perda da modulação do SRAA (SILVA *et al*., 2003).

No que se diz respeito ao peso no nascimento alguns estudos tem divergidos entre si. Beauséjour et al., (2003) demonstraram que quando a sobrecarga de sódio inicia-se a partir do 15º dia gestacional em ratas, a prole nasce com o peso reduzido. No entanto, quando a sobrecarga de sódio materna inicia-se antes da concepção o peso no nascimento não é alterado (SILVA *et al*., 2003; CARDOSO *et al*., 2009) no entanto o ganho ponderal de ratos submetidos à sobrecarga de sódio perinatal apresenta se diminuído (SILVA *et al*., 2003).

Dados do nosso laboratório evidenciam que a alta ingestão materna de sódio durante o período perinatal, traz repercussões a prole adulta como expansão de volume plasmático, proteinúria, elevação do estresse oxidativo renal e diminuição do

ritmo de filtração glomerular (CARDOSO *et al*., 2009). Portanto, apesar de, a nefrogênese não ser comprometida (BALBI *et al*., 2004; CARDOSO *et al*,.2009) na prole de mães submetidas a sobrecarga de sódio, a função bem como, o desenvolvimento das estruturas foram comprometidas. Neste contexto ratos adultos submetidos à sobrecarga de sódio durante o período perinatal, apresentam comprometimento do tecido renal como glomeruloesclerose, lesões túbulo intersticiais e infiltrado inflamatório (MARIN *et al*., 2008) o que pode estar correlacionado com ativação inadequada do SRAA intrarenal (SILVA *et al.*, 2003) uma vez que a ANG II é pró-inflamatória e pró-fibrotica (ÉGIDO, 1996; MEZZANO *et al*., 2001).

2.3 Repercussões da sobrecarga de sódio sobre o SRAA e desenvolvimento renal fetal

A nefrogênese em humanos inicia-se a partir da 5º semana e conclui-se antes do nascimento por volta da 38º semana de gestação. Em ratos, tem inicio na 12º dia de vida embrionária, e diferente da nefrogênese em humanos, conclui-se entre 13-15º dia de vida pós natal (REEVES *et al*., 1978; NIGAM *et al*., 1996). Vários estudos têm evidenciado que o SRAA é de suma importância para o desenvolvimento do tecido renal fetal (TUFRO-MCREDDIE *et al*., 1995). Estudos têm demonstrado que o bloqueio farmacológico da enzima conversora de angiotensina ou o bloqueio de receptores AT_1 altera a formação do tecido renal e assim compromete a função renal (FRIBERG *et al*., 1994; TUFRO-MCREDDIE *et al*., 1995; DAIKHA-DAHMANE *et al*., 2006). Camudongos que tiveram mutação no gen da enzima conversora de angiotensina apresentam alterações renais, como distúrbios na formação nefrovascular (HILGERS *et al*., 1997), número reduzidos de glomérulos maduros, glomérulos com hipotrofia, dilatação tubular, fibrose intersticial renal e dilatação pélvica (NIIMURA *et al*., 1995). Estudo realizado com feto humano onde a mãe foi tratada com antagonista de receptor AT_1 a histologia renal mostrou disgenesia tubular, comprometimento da *vasa recta* e distúrbio do desenvolvimento do epitélio tubular (DAIKHA-DAHMANE *et al*., 2006). Em ratos, a prole de mães que são tratadas durante a gestação e lactação com losartan apresenta dilatação pélvica, inflamação do tecido renal e irregularidades do parênquima renal (SPENCE *et al*., 1995). Ratos neonatos tratados com enalapril apresentam atrofia papilar renal, bem como redução da expressão da proteína aquaporina 2 na medula interna, o que leva

a perda da capacidade de concentração urinária (GURON *et al*., 1999). Adicionalmente, apresentam também inflamação túbulo-intersticial, atrofia papilar e dilatação pélvica (FIRBERG *et al*., 1994). Disfunções da reabsorção tubular proximal e alterações na hemodinâmica renal também foram observadas nesses ratos, como redução da taxa de filtração glomerular e do fluxo plasmático renal, os quais se apresentam reduzidos em aproximadamente 15% (GURON *et al*., 1998).

2.4 Repercussões da sobrecarga de sódio sobre transportadores de sódio no epitélio tubular

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 (α1, α2, α3 e α4) expressas em vários tecidos, sendo a forma predominante nas células epiteliais renal a isoforma α1 (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 hormônios como dopamina e ANG II 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 a 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 base dos microvilos, enquanto no ramo ascendente da alça de Henle e no túbulo distal ocorre retração do cotransporte $Na^+K^+2Cl^-$ e de canais de sódio (ENaC), respectivamente, para 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 ainda retração 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 dopamina é um hormônio natriurético que é produzido, sobretudo no sistema nervoso central, e também no epitélio tubular renal onde tem ação autócrina/parácrina. Diante de sobrecarga de sódio a excreção urinária de dopamina apresenta-se elevada (ALEXANDER *et al*., 1974), o que parece ser um indicador de produção aumentada pelo rim. A dopamina age em diferentes segmentos do néfron (MEISTER *et al*., 1989; BERTORELLO *et al*., 1990) mais caracteristicamente através de receptores D1, uma vez que alterações estruturais nestes receptores têm sido associadas com retenção de fluido na hipertensão essencial (JAITOVICH *et al*., 2010). A ativação destes receptores no túbulo proximal e ramo espesso ascendente da alça de Henle resulta em inibição de (Na^+-K^+)ATPase (BERTORELLO *et al*., 1990).

Por outro lado, a ANG II é um hormônio anti-natriurético que atua no túbulo proximal através de dois receptores, AT_1 e AT_2 (GILDEA, 2009). A ANG II 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 ela tem efeito oposto (HARRIS *et al*., 1977). Assim, em concentrações fisiológicas, a ANG II aumenta a reabsorção tubular proximal de sódio. Quando a pressão de perfusão renal apresenta-se diminuída, a ANG II 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 a aumento da concentração de Na^+ intracelular e subsequentemente aumentando a atividade da (Na^++K^+)ATPase (REILLY *et al*., 1995; WONG, *et al*., 1996). A ANG II tem ainda um efeito direto sobre a (Na^++K^+)ATPase, através da diminuição da sensibilidade a ouabaína bem como alterações no seu estado de conformação e fosforilação (YINGST *et al*., 2004).

Quando os mecanismos moleculares e humorais não conseguem manter o balanço entre natriurse e antinatriurese, a elevada ingestão de sódio pode induzir hipertensão (JAITOVICH *et al*., 2010). Ratos espontaneamente hipertensos (SHR) desenvolvem hipertensão entre 4-6º semanas de vida. É possível que a hipertensão

desenvolvida por esta linhagem de ratos seja, em parte, devida a atividade aumentada da (Na^++K^+)ATPase (GARG *et al*., 1985) no túbulo proximal. Alem disso tem sido observado que a capacidade de inibição da atividade da (Na^++K^+)ATPase pela dopamina, em ratos SHR, esteve diminuída quando comparado com Wistar Kyoto. Há também alterações na expressão e distribuição das subunidades da (Na^++K^+)ATPase como α1 e γ (HINOJOS *et al*., 2004). O aumento da expressão protéica da subunidade γ aumenta a afinidade da (Na^++K^+)ATPase à molécula de ATP (ARYSTARKHOVA *et al*., 1999; THERIEN *et al*., 1999) e leva a aumento da reabsorção de sódio no túbulo proximal de ratos 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 de ratos SHR (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 participa da homeostase de sódio e apresenta as peculiaridades de ser insensível a ouabaína e sensível a furosemida. Na^+-ATPase é responsável pelo ajuste fino da reabsorção de sódio no túbulo proximal e sua atividade é regulada pela ANG II e seus metabolitos biologicamente ativos como angiotensina 4 e (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 ANG II aumentam a atividade da Na^+-ATPase, enquanto elevadas concentrações têm efeito oposto. Em ratos SHR foi demonstrado que a atividade da Na^+-ATPase está aumentada na 14 semana de vida, momento na qual a hipertensão está estabelecida (QUEIROZ-MADEIRA *et al*., 2009). No entanto, nessa mesma linhagem foi demonstrado que a ANG II diminui a atividade da Na^+-ATPase e que esta inibição pode estar relacionada com a ativação de receptores AT_2 (QUEIROZ-MADEIRA *et al*., 2009).

3. Possibilidades de Reprogramação de Eventos Moleculares e Fisiológicos

A hipertensão programada durante a vida intra uterina, em ratos, pode ser prevenida quando a ingestão dietética de sódio é muito baixa ou exacerbada quando a ingestão de sódio é elevada (STEWART *et al*., 2009). A hipertensão programada durante a vida intrauterina é também prevenida quando se utiliza inibidores de enzima conversora durante curtos intervalos de tempo (MANNING *et al*., 2004).

Ratos SHR que receberam antagonista de receptores AT_1, a partir da 4º até a 10º semana de vida, apresentaram níveis pressóricos reduzidos mesmo após o fim do tratamento (QUEIROZ-MADEIRA *et al*., 2009). Stewart et al., (2005) demonstraram que ratos que são programados pela desnutrição materna, durante o período do desenvolvimento, para desenvolver hipertensão, quando tratados com tempol, um mimético da superóxido dismutase, ou com um imunosupressor, também durante curto intervalo de tempo, têm a hipertensão prevenida. Neste estudo, os autores enfatizam o papel 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 ANG II programada pela desnutrição materna durante a gravidez (VIEIRA-FILHO *et al*., 2010).

Com base nas evidências de que os distúrbios moleculares e fisiológicos programados durante o período perinatal podem ser revertidos, neste trabalho investigamos, em ratos adultos, as repercussões da sobrecarga de sódio perinatal sobre possíveis alterações nos transportadores de sódio renal. Além disso, investigamos se o tratamento com enalapril, um inibidor de enzima conversora, administrado após o desmame, durante três semanas, previne as alterações produzidas pela sobrecarga de sódio durante o período perinatal.

OBJETIVOS:

4.1 Geral:

Avaliar, em ratos, os efeitos da sobrecarga de sódio durante o período perinatal sobre a pressão arterial e função tubular proximal e verificar se o tratamento com enalapril, após o desmame durante três semanas, reverte as possíveis alterações moleculares induzidas pela alta ingestão de sódio materna.

4.2 Especifícos:

1) Avaliar a pressão arterial média.
2) Avaliar a expressão protéica da $(Na^+ + K^+)$ATPase em membrana basolateral do túbulo proximal.
3) Avaliar a atividade de transportadores de sódio da membrana basolateral do túbulo proximal, Na^+-ATPase e $(Na^+ + K^+)$ATPase.
4) Avaliar os componentes do SRAA, tais como a expressão dos receptores AT_1 e AT_2 e a atividade de seus sinalizadores intracelulares PKC e PKA.

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Prenatal Na^+ overload turns on programming of increased proximal Na^+ reabsorption and onset of hypertension in adult rats, and enalapril treatment after weaning turns it off

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Running head: Programming of adult hypertension by perinatal Na^+ overload

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The renal mechanisms underlying perinatal Na^+ overload-induced hypertension were investigated, as were the effects of short treatment with enalapril in terms of the reprogramming of molecular alterations in the kidney and the onset of hypertension. 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) 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 activity without alterations in α-subunit expression. Ouabain-insensitive Na^+-ATPase activity remained unchanged but its response to angiotensin II (ANG II) was lost. Protein kinase C (PKC), cAMP-dependent protein kinase (PKA) and renal thiobarbituric acid reactive substances (TBARS) increased, AT_2 receptor expression decreased and AT_1 receptor expression remained unchanged. Early treatment with enalapril reduced the activities of both pumps, and reduced PKC and PKA activities depending on whether the offspring were exposed to high Na^+ perinatally. In addition, treatment with enalapril lowered AT_2 receptor expression and increased local TBARS. Reduced PKA activity in enalapril-treated saline offspring was accompanied by a recovery of the stimulatory, but not the inhibitory, response to ANG II. The ninety day old saline offspring had higher blood pressure than controls and early enalapril administration blocked the onset of hypertension, indicating reprogramming of $(Na^+ + K^+)$ATPase. Enalapril was beneficial in decreasing Na^+ reabsorption and preventing hypertension in adult offspring. However, side effects including down-regulation of PKA, PKC and AT_2 receptors, and increasing TBARS could impair renal function in later life.

Keywords: Na^+ overload; renal $(Na^+ + K^+)$ATPase; hypertension; renal programming; enalapril

HIGH Na^+ INTAKE IS an emerging reality of modern society in developed and developing countries, owing particularly 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 rats are exposed to Na^+ overload from conception to weaning, as adults the offspring exhibit plasma renin activity that 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 (4). Therefore, perinatal Na^+ overload leads to renin angiotensin system (RAS) over-activity during adulthood. 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.

Kidney development in the rat ends at approximately postnatal day 12 (6), and pharmacological inhibition of RAS during this period causes severe alterations in renal structure and function (7-9). In humans, pharmacological inhibition of RAS during the second and third trimesters of pregnancy causes renal anomalies in offspring (10-12). However, there is evidence that a short-term inhibition of RAS after weaning in rats could reverse prenatal programmed hypertension induced by maternal undernutrition (13). In addition, it has been demonstrated that early maternal postnatal treatment of rats subjected to prenatal undernutrition with α-tocopherol prevents alterations in proximal tubule Na^+ transporters (14). The beneficial effects of inhibiting RAS after weaning in rats show that the window of opportunity for imprinting molecular changes that affect renal function in adult life lasts beyond the conclusion of nephrogenesis and weaning (13, 14). Therefore, various related early pathological processes can be reprogrammed in different ways to achieve normal profiles in adult life.

Elevated reabsorption of Na^+ in the proximal tubule has been demonstrated in primary hypertension (15) and spontaneously hypertensive rats (SHR) (16). Overactive renal ATP-dependent Na^+ transporters, particularly the ouabain-resistant Na^+-ATPase resident in the basolateral membrane of proximal tubule cells, has been demonstrated in SHR (17). Perinatal Na^+ overload leads to RAS overactivity associated with hypertension and renal complications, but the molecular mechanisms underlying such complications have not been investigated in renal membranes where active Na^+ transport occurs. The present study was designed to determine whether Na^+ overload during the perinatal period imprints changes in proximal tubule ATP-driven Na^+ transporters, $(Na^+ + K^+)$ATPase and Na^+-ATPase. 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), and the expression and activity of protein kinases C and A.

MATERIALS AND METHODS

Animal care

Animal experimental procedures described in this study were approved by the Committee for Ethics in Animal Experimentation of the Federal University of Pernambuco, and carried out in accordance with Committee guidelines.

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 Biotecnology (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-rabbit antibody and the ECL™ Western blotting system were obtained from GE Healthcare. ^{32}Pi was purchased from the Brazilian Institute of Energy and Nuclear Research (São Paulo, SP, Brazil). (γ-^{32}P)ATP was synthesized as in (18). The commercial urea kit was obtained from Labtest (Lagoa Santa, MG). 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 a maternal 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. Mating was carried out at 90 days of age. The rats (dams and offspring after weaning) were provided with a balanced commercial rodent chow (Purina Agribands). The control group (C, n = 18) was composed of the offspring of mothers that had consumed tap water throughout the study. 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 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 with tap water supplemented with enalapril maleate (E, 100 mg/l) for three weeks, and with pure tap water from then to the time of the experiments; these subgroups

were denoted by CE (*n* = 8) and SE (*n* = 11), respectively. Each rat exposed to enalapril was exposed to an average of 4 mg per day. All experiments were carried out on animals aged 90 days.

Blood pressure and metabolic studies

Blood pressure was measured in awake animals using tail-cuff plethysmography (IITC Life Science B60-7/16", Life Science Instruments, Woodland Wills, CA). Twenty-four hour urine samples were obtained to measure proteinuria, urea and dietary intake. Twenty-four hours after completion of the metabolic studies, animals were decapitated and the kidneys and liver removed. The most superficial region of the cortex (cortex corticis) of the kidney was removed to isolate the tubular plasma membranes (see below); the remaining tissue was used to evaluate thiobarbituric acid reactive substances (TBARS) as a measure of lipid peroxidation and therefore renal oxidative stress. The liver was isolated for additional assessment of oxidative stress.

Evaluation of tissue oxidative stress

Lipid peroxidation was assessed in the liver and renal medulla by measuring TBARS according to (19). Tissues, 1 g, were homogenized with 5 ml KCl 1.15% in an ice bath. Two ml 0.375% thiobarbituric acid diluted with 15% TCA was added to each ml of homogenate. The tubes were sealed and heated to 100ºC 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 obtained as previously described (20) from the cortex corticis, a region where more than 90% of the cell population corresponds to the proximal tubules (21, 22). 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. Thin transverse sections (0.5 mm) were cut using a Stadie-Riggs microtome and carefully dissected with iridectomy scissors to eliminate contamination with internal regions. The fragments were homogenized in 4 ml of a solution containing 250 mM sucrose, 10 mM HEPES-Tris (pH 7.4), 2 mM EDTA, 0.15 mg/ml trypsin inhibitor and 1 mM PMSF per gram of tissue in an ice bath. The homogenate was centrifuged at 755 *g* (15 min) to sediment cell debris and nuclei; the resulting supernatant was centrifuged at 8,500 *g* (20 min) and at 35,000 *g* (45 min). The final sediment was resuspended in 250 mM sucrose, aliquoted into tubes and stored at −20ºC. Protein content

was determined using the Folin phenol method (23) with BSA as a standard, using 2.5% (w/v) SDS to solubilize integral membrane proteins. Controls for enrichment with basolateral membranes and for minimal residual contamination with other intracellular membranes were as previously described (20). The final fraction contained apical membranes but at a lower yield than the starting homogenate, as revealed using alkaline phosphate assays (20). However, ATP-driven Na^+ transporters are exclusively located in the basolateral aspect of the cell membrane. Therefore, no attempt was made to fractionate the samples further. The Percoll gradient method was used to separate brush border and basolateral membranes as used previously for porcine and ovine kidneys (24, 25), as it requires the reduced number of rats 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. In (Na^++K^+)ATPase assays, the membranes (0.1 mg/ml final concentration) were pre-incubated with or without 2 mM ouabain in 0.1 ml water at 37^{o}C for 20 min. The assay mixtures 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 hydrolysis reaction was started by adding ATP (5 mM) and KCl (24 mM), 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 Pi released in the absence and presence of ouabain. Released Pi was spectrophotometrically measured in a 0.2 ml aliquot of the supernatant obtained after centrifugation of the charcoal suspension at 1,500 *g* for 5 min.

The ouabain-insensitive Na^+-ATPase activity was measured using (γ-^{32}P)ATP (~0.03 MBq/µmol) or unlabelled ATP; both methods produced identical results ($P > 0.05$) and the measurements were grouped for final statistical analysis. The activity was calculated from the difference between the Pi or ^{32}Pi released in the absence and presence of 2 mM furosemide. The reaction was started by adding 5 mM 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 by adding two vol 0.1 M HCl-activated charcoal. Released Pi was measured by liquid scintillation counting (Packard) or by spectrophotometry in a 0.2 ml aliquot of the supernatant obtained after centrifugation of the charcoal suspension (1,500 *g* for 5 min).

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

The protein kinase activities were analyzed by measuring the incorporation of the γ-phosphoryl group of (γ-^{32}P)ATP (specific activity ~1 MBq/nmol) into histone in the absence and presence of the specific PKC and PKA inhibitors, 100 nM calphostin C and 10 nM $PKAi_{5-24}$, as described (24). 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 membrane protein. After two min, the reaction was stopped by adding 0.1 ml 40% TCA and the samples were immediately placed on ice. After vigorous stirring, an aliquot of 0.1 ml 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). The radioactivity was quantified using a liquid scintillation counter.

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 on membranes (25). Isolated membranes (1 mg/ml) were initially solubilized in a sucrose solution containing 0.01% CHAPS for 30 min at room temperature. Primary polyclonal antibody (1:400 dilution) was mixed with protein A-agarose, gently stirred for 20 min and supplemented with an equal volume of BSA (1 mg/ml) in 0.01% CHAPS. This mixture was added to the isolated membrane samples. After constant stirring overnight at 4ºC, the samples were centrifuged at 1,000 *g* for four min. The supernatant was retained as an important control for the immunoprecipitation procedure. The pellet was washed three times with Tris-buffered saline (TBS) and heated to 100ºC in a water bath for four min with 40 μl SDS-PAGE sample buffer. After final centrifugation at 10,000 *g* for two 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 immunodetected directly in the membranes or immunoprecipitates, respectively, using specific antibodies. The 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 one hour. The membranes were probed with the corresponding primary antibodies (1:500 dilution) for one hour at room temperature under gentle stirring, washed three times with TBS containing 0.1% Tween 20, exposed to the secondary antibody, washed and visualized using ECL™.

Analytical Methods

Urinary protein and urea were measured using the Folin phenol method (23) and a commercial kit, respectively.

Statistical Analysis

Differences among groups were analyzed using a one way ANOVA test followed by a Student-Newman-Keuls post test. One-way ANOVA followed by Tukey's test analysis was used to compare the responses of Na^+-ATPase to ANG II within groups. GraphPad Prism 5 software (Version 5.01, GraphPad Software, Inc.) was used for all statistical analyses. The statistical differences were considered significant at $P < 0.05$.

RESULTS

Body weight evolution in the offspring

Maternal Na^+ overload during gestation did not affect the birth weight of offspring, and Na^+ overload during lactation did not affect the body weight of progeny at weaning (Table 1). Enalapril, which inhibits the angiotensin I-converting enzyme (ACE), was used in the present study for three weeks after weaning and had no influence on the body weight of adult offspring. However, by 70 and 90 days of age, the body weight of progeny exposed to high Na^+ (S and SE) during the perinatal period was lower than both control groups (C and CE) and enalapril administration had no effect (Table 1). The dietary intake after weaning was similar among the four groups (data not shown). Therefore, urinary urea excretion was investigated as an index of metabolic turnover, and demonstrated no statistical difference among groups (Table 1).

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

Fig. 1 demonstrates that perinatal Na^+ overloading did not affect expression of the α-subunit of $(Na^+ + K^+)$ATPase in basolateral membranes of proximal tubules from 90 day old offspring. However, treatment with enalapril three weeks after weaning led to a relative decrease in expression of the α-subunit in the CE and SE groups. Nevertheless, when enzyme activity was measured, perinatal Na^+ overload programmed elevated activity of $(Na^+ + K^+)$ATPase (Fig. 2; compare S vs. C group). Treatment with enalapril for three weeks after weaning reprogrammed this enzyme, resulting in activity at levels exhibited by the control group (compare SE vs. C and SE vs. S). Enalapril lowered $(Na^+ + K^+)$ATPase activity in control animals by approximately 50% (compare CE vs. C). The activity of ouabain-

insensitive Na^+-ATPase (Fig. 3) was not affected by perinatal Na^+ overload (compare S vs. C group). However, enalapril reduced ouabain-insensitive Na^+-ATPase activity in control rats and the progeny of Na^+-overloaded mothers (Fig. 3; compare CE vs. C and SE vs. S).

Na^+-ATPase activity was measured in the presence of rising ANG II concentrations (Fig. 4). The effect of the peptide was biphasic in control rats; 10^{-12} M ANG II significantly increased Na^+-ATPase activity, and high concentrations led to a progressive inhibition of the previously stimulated activity (Fig. 4, upper left panel). The effect of ANG II was lost in the S group at all concentrations assayed (Fig. 4, upper right panel). Enalapril lowered the activity of Na^+-ATPase in the absence of ANG II in the control group (CE), with preservation of the stimulatory effect at 10^{-12} M (Fig. 4, lower left panel). In the SE group, enalapril treatment restored the basal value of Na^+-ATPase activity and stimulation by 10^{-12} M ANG II (Fig. 4, lower right panel). However, in both offspring groups, enalapril suppressed the inhibitory effect of high ANG II concentrations.

PKC and PKA activities

Stimulatory effects of low ANG II concentrations on $(Na^+ + K^+)$ATPase and Na^+-ATPase is mediated by PKC (26, 27). In addition to being a subcellular target of ANG II in the signaling cascade originating from ANG II receptors, PKC is activated by superoxide anions (28), and renal oxidative stress is increased during chronic juvenile Na^+ overload (29). In the present study, PKC was increased in adult rats from mothers that had been subjected to Na^+ overload (Fig. 5). Enalapril treatment after weaning did not affect PKC activity in control rats (CE) and strongly decreased PKC activity in prenatally Na^+ overloaded rats (SE) (Fig. 5). PKA, which counteracts the PKC-mediated stimulatory effect on Na^+-ATPase activity (27), was increased by more than 100% in rats programmed by perinatal Na^+, and treatment with enalapril reduced its activity in both groups to comparable low levels (Fig. 6).

AT_1 and AT_2 receptor density

To investigate whether Na^+-induced and enalapril-mediated changes in Na^+-stimulated ATPases activities (Figs. 2 and 3) and kinase activities (Figs. 5 and 6) were due to upstream alterations in the RAS cascade, the expression of AT_1 and AT_2 receptors were analyzed. Perinatal Na^+ overload and three-week treatment with enalapril after weaning programmed a reduction in the expression of AT_2 receptors in adult offspring (Fig. 7*A*) and their effects appeared to be additive (SE). However, maternal Na^+ overloading and enalapril had no effect (alone or combined) on AT_1 receptor expression (Fig. 7*B*).

Proteinuria and renal oxidative stress

In adult offspring, enalapril prevented proteinuria programmed by perinatal Na^+ overload (SE vs. S in Table 2). Paradoxically, enalapril increased proteinuria in control animals (compare CE vs. C). Chronic Na^+ overload after weaning increases renal oxidative stress (28) and this is associated with inflammatory processes that lead to proteinuria (29, 30). Therefore, whether perinatal Na^+ overload could imprint increased oxidative stress in the adult kidney was investigated. TBARS levels in the kidneys of the S group were higher than in the C group (Table 1); enalapril did not prevent the increase in lipid peroxidation in rats perinatally programmed by high Na^+ exposure (compare SE vs. S) but increased this process in controls (compare CE vs. C). TBARS were evaluated in liver as a control for the lipid peroxidation study, and they remained unchanged in the S group when compared to controls. Therefore, enalapril led to increased lipid peroxidation in the CE and SE groups.

Blood pressure

The mean blood pressure (MBP) of animals in the S group was higher than in the C group, and treatment with enalapril prevented an increase in blood pressure (SE) without affecting the control animals (CE) (Fig. 8).

DISCUSSION

The present study describes novel results that partially elucidate the renal molecular mechanisms underlying hypertension in adult offspring that have been programmed by perinatal Na^+ overload. Furthermore, this research demonstrates that short-term treatment with enalapril after weaning can reprogram the Na^+ overload-induced renal alterations in progeny and the onset of hypertension. Increased $(Na^+ + K^+)$ATPase activity in the basolateral membranes of proximal tubules (Fig. 2) could account for the increased blood pressure observed in rats subjected to perinatal Na^+ overload (Fig. 8), as this pump is responsible for the majority of proximal Na^+ reabsorption [for a review see (31)]. Post-translational alterations appear to be involved, as the expression and targeting of the pump were preserved in rats perinatally exposed to high Na^+ (Fig. 1). The permanent up-regulation of the pump evident in Fig. 2 could be the result of an erroneous adaptative process involving nuclear and cytosolic factors and enzymes such as PKC (32, 33), activated as a consequence of low level apical Na^+ entry. This incorrect signal could have been elicited by the retraction of brush border Na^+ transporters, viewed as a response to the increased luminal Na^+ evident during Na^+ overloading (34). Reactive oxygen species might participate in this programming process, as

evidenced by the more than 40% increase in local TBARS in the S group (Table 2). Na^+ overload can increase $O_2^{\bullet-}$ in the tubular epithelium owing to increased metabolic (respiratory) demand for Na^+ reabsorption after increased delivery of the cation to the basolateral Na^+ pumps, and other mechanisms appear to be involved. Acute (35, 36) and chronic (37) Na^+ overload could lead to exacerbated local $O_2^{\bullet-}$ because of upregulation of AT_1 (38) or downregulation of AT_2 (39, 40) receptors. This is evident in the present study: a persistent abnormal imprint on AT_2 receptors caused a reduction in their expression in rats perinatally exposed to high Na^+ (Fig. 7*A*) and an increased level of TBARS (Table 2).

Reduced $(Na^+ + K^+)$ATPase expression (Fig. 1) and restoration of control $(Na^+ + K^+)$ATPase activity (Fig. 2) by treatment with enalapril after weaning was indicative of local RAS (41) playing a crucial role in the programming of signaling pathways that culminate in the up-regulation of $(Na^+ + K^+)$ATPase activity in adult life. Interestingly, enalapril programmed a decrease in AT_2 receptor expression (Fig. 7*A*) and increased local oxidative stress (Table 2), side effects that indicate parallel actions of the drug beyond its action in reprogramming $(Na^+ + K^+)$ATPase turnover (Fig. 2). The additive effects of perinatal Na^+ overload and enalapril treatment on AT_2 receptor expression (Fig. 7*A*) clearly shows there are separate pathways in which RAS participate that affect renal molecular programming and reprogramming. The effects of enalapril treatment after weaning support the view that renal programming continues after nephrogenesis is completed (6). As previously mentioned, down-regulation of AT_2 receptors and augmented $O_2^{\bullet-}$ are clearly associated with renal programming (39, 40). The influences of Na^+ overload and enalapril treatment are not additive with respect to effects on renal TBARS (Table 2). Therefore, they are likely to impact on a common final enzymatic target in tubule cells, thereby impairing $O_2^{\bullet-}$ detoxification.

Renal ouabain-insensitive Na^+-ATPase is modulated *in vitro* by hormones and autacoids that participate in the physiological regulation of extracellular fluid (17, 27, 42, 43), but its activity remained unaltered in the membranes of animals in the S group (Fig. 3). However, Na^+ overload programmed an altered response to its physiological activator ANG II (compare panels C and S in Fig. 4). The lack of sensitivity to ANG II over a wide range of concentrations could be due to the Na^+ overload-induced programming leading to up-regulation of PKA activity (Fig. 6), as the activity of its physiological activator, PKC (42), was also increased (Fig. 5). A possible link between PKA activation and the unresponsiveness of Na^+-ATPase to ANG II could be an alternative route for RAS mediated by ACE2 (44), causing local Ang-(1-7) formation. Acting through MAS receptors (45), this peptide would imprint constitutive activation of PKA, leading to the blockade of Na^+-ATPase activation by 10^{-12} M ANG II. Recently, it was demonstrated that Ang-(1-7) is formed in the basolateral membranes of proximal tubule cells by an ACE2-mediated pathway (46) that can counteract

ANG II/AT_1 effects on ion transport (25). Therefore, the loss of the inhibitory effect of high ANG II concentrations in animals from the S group presented in Fig. 4 is likely to be due to a programmed decrease in the expression of AT_2 receptors (Fig. 7*A*), the first step in the ANG II-associated cascade that culminates in the inhibition of Na^+-ATPase caused by high concentrations of the peptide (17).

The actions of enalapril on Na^+-ATPase activity (Fig. 3), its regulation by ANG II (Fig. 4) and the activities of PKC and PKA (Fig. 5) indicate the drug affects branched pathways that participate in programming and reprogramming molecular modifications at the level of renal Na^+ transport. Moreover, the results demonstrate that perinatal exposure to high Na^+ modifies the response to enalapril treatment after weaning as detailed: (i) Enalapril inhibited Na^+-ATPase activity to varying extents in the CE and SE groups (Fig. 3) and this could be due to a Na^+-induced local RAS hypereactivity in the perinatally programmed offspring; (ii) Enalapril blocked the inhibitory effect of high ANG II concentrations on Na^+-ATPase in the absence of Na^+ overload programming (compare panels CE and SE in Fig. 4), because the programming reduced AT_2 receptor expression (Fig. 7*A*); (iii) Enalapril preserved the normal activity of PKC in controls but caused an accentuated inhibition of this activity in programmed rats (Fig. 5); (iv) Enalapril treatment programmed an accentuated (70%) inhibition of PKA activity irrespective of whether the progeny were programmed by perinatal Na^+ (Fig. 6). This pleiotropic ensemble of responses can be explained by the complex interacting pathways and the large number of effectors targeted during the signaling cascade concerning local RAS (41).

Markedly increased mean blood pressure in adult offspring (Fig. 8) that was associated with the programming of increased renal (Na^++K^+)ATPase activity (Fig. 1), emerged as the systemic consequence of the impact of perinatal Na^+ overload on kidney targets. Increased PKC (Fig. 5) and loss of Na^+-ATPase inhibition by high local ANG II expression (Fig. 4) could increase the transepithelial flux of fluid (26, 42), expansion of extracellular compartments and an increase in bodily Na^+ content (for a review see 47). These programmed modifications contribute to late onset and maintenance of hypertension. Enalapril treatment caused reprogramming of normal arterial pressure in perinatally Na^+ overloaded rats but had no effect in control animals (Fig. 8), and this indicates a specific influence of enalapril on intrarenal RAS only when the animals were programmed by maternal Na^+ overload and the Ang II signaling cascade is altered, having – for example – permanent modifications in the kinases and receptors as here evidenced. Thus, inhibition of intrarenal ACE with direct repercussion on proximal tubule (Na^++K^+)ATPase in a short window of growing after weaning appears to restore normal tensional values in young adult rats, over-riding possible effects of other renal molecular modifications. Manning and Vehaskari (13) proposed a critical time-period for programming hypertension that could be

modified later. The anti-hypertensive effect of enalapril demonstrated that: (i) a short period after weaning is the time window for modulation; (ii) simultaneous reprogramming of renal RAS, even initially benefic, can later negatively affect renal transporting and regulatory molecular machinery.

In conclusion, perinatal Na^+ overload programs increased blood pressure during adult life owing to an increase in proximal Na^+ reabsorption. Early treatment with enalapril reprograms and reduces the majority of Na^+ reabsorption that acts on basolateral (Na^++K^+)ATPase and prevents the onset of hypertension. However, the side effects of treatment with enalapril, particularly those associated with down-regulation of AT_2 receptors, could have an overall negative impact on renal function.

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DISCLOSURES

The authors have no conflicts of interest.

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Figure Legends

Fig. 1. Programming effects of perinatal Na^+ overload and early treatment with enalapril on (Na^++K^+)ATPase expression in basolateral membranes from proximal tubules. (*A*) Representative immunoblots. (*B*) Densitometric representation of the immunoblots. Values are means ± SE (n = 4) of the densitometric determinations after correction for protein loading. The values of the control band were taken as 100 % and those from the experimental groups in the same gel were expressed as a fraction of the corresponding control. C and S are the offspring from control and perinatally (in utero + lactation) Na^+-overloaded mothers, respectively, and CE and SE correspond to the previous two groups treated with enalapril for three weeks after weaning. Different lowercase letters above the bars indicate significant differences ($P < 0.05$).

Fig. 2. Programming of (Na^++K^+)ATPase activity by perinatal Na^+ overload and early enalapril treatment. The offspring groups (C, S, CE and SE) are as described in the legend to Fig. 1. Values are means ± SE (n = 5–7). Different lowercase letters above the bars indicate statistically different values ($P < 0.05$).

Fig. 3. Programming effects of perinatal Na^+ overload and early enalapril treatment on the ouabain-insensitive Na^+-ATPase activity from proximal tubule basolateral membranes. C, S, CE and SE have the same meanings as described in the legend to Fig. 1. Values are means ± SE (n = 14–19). Different lowercase letters above the bars indicate statistically different values ($P < 0.05$).

Fig. 4. Programming effects of perinatal Na^+ overload and early treatment with enalapril on the responsiveness of Na^+-ATPase activity to ANG II. Panels C (upper left) and S (upper right) correspond to the offspring of control and perinatally (in utero + lactation) Na^+-overloaded mothers. Panels CE (lower left) and SE (lower right) correspond to the previous two groups treated with enalapril for three weeks after weaning. Na^+-ATPase activity was assayed in the presence of the ANG II concentrations shown on the abscissae. Values are means ± SE (n = 5–9 depending on the group). Different lowercase letters above the circles indicate differences ($P < 0.05$) by comparing mean values within and among figures. One-way ANOVA followed by Tukey's test analysis was used to compare the data within each group. One-way ANOVA with a Student-Newman-Neuls test post hoc analysis was used to compare the data among groups.

Fig. 5. Programming by perinatal Na^+ overload and early enalapril treatment affects protein kinase C (PKC) activity in proximal tubule cell membranes. C, S, CE and SE have the same meanings as described in the legend to Fig. 1. Values are means ± SE (n = 3). Different lowercase letters above the bars indicate statistically different values ($P < 0.05$).

Fig. 6. Programming of cAMP-dependent protein kinase (PKA) activity. C, S, CE and SE have the same meanings as described in the legend to Fig. 1. Values are means ± SE (n = 4–5). Different lowercase letters above the bars indicate statistic differences ($P < 0.05$).

Fig. 7. AT_2 receptors (*A*) but not AT_1 receptors (B) in proximal tubule cells of adult offspring are programmed by perinatal Na^+ overload. Upper panels: representative immunodetections. Lower panels: densitometric representations. C, S, CE and SE on the abscissae have the same meaning as those described in the legend to Fig. 1. Values are means ± SE of densitometric determinations after correction for protein loading. The control values were taken as 100% and those from experimental groups in the same gel were expressed as a fraction of the corresponding control (n = 3–5 for AT_2; n = 5 for AT_1). Different lowercase letters above the bars in the lower panels indicate statistically different values ($P < 0.05$).

Fig. 8. Perinatal Na^+ overload-induced hypertension in adult offspring: reprogramming by treatment with enalapril for three weeks after weaning. Capital letters on the abscissa indicate the groups described in the legend to Fig. 1. Values are means ± SE of eight or nine measurements using various rats, which were recorded as described in the Materials and Methods section. Different lowercase letters above the bars indicate statistically different values ($P < 0.05$).

Table 1. *Body weight evolution of the offspring*

	C	S	CE	SE
Body weight at birth, g	6.41 ± 0.18	6.17 ± 0.19	-	-
Body weight at weaning, g	49 ± 2	48 ± 3	-	-
Body weight at *day* 70, g	317 ± 6	282 ± 8*	306 ± 5	271 ± 5‡
Body weight at *day* 90, g	349 ± 7	305 ± 12*	350 ± 6	306 ± 9‡
Urinary urea, mmol/24 h	69 ± 4	72 ± 6	76 ± 4	75 ± 4

Values are means ± SE. * $P < 0.05$ vs. C. ‡ $P < 0.05$ vs. CE.

Table 2. *Effects of perinatal Na^+ overload and early enalapril treatment on proteinuria and renal malonydialdehyde*

	C	S	CE	SE
Proteinuria, mg/24h	11.6 ± 1.5	16.9 ± 1.7*	16.0 ± 2.2*	12.4 ± 1.6 ‡ †
Renal TBARS, nmol MDA/g	5.3 ± 0.7	8.8 ± 0.6*	8.0 ± 0.4*	8.3 ± 0.6 *
Hepatic TBARS, nmol MDA/g	4.4 ± 0.1	4.6 ± 0.1	6.3 ± 0.1*	5.6 ± 0.1* † ‡

Values are means ± SE (*n* indicated in text, *Animal groups* subsection). TBARS, thiobarbituric acid reactive substances; MDA, malonydialdehyde. * $P < 0.05$ vs. C. † $P < 0.05$ vs. S. ‡ $P < 0.05$ vs. CE.

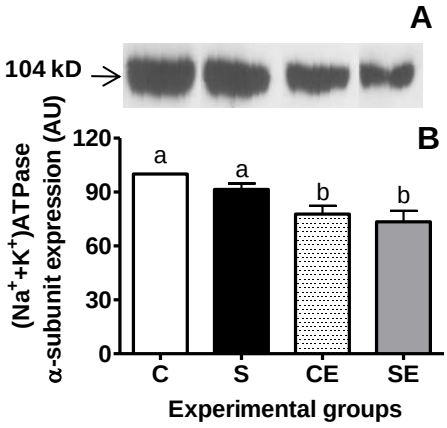
A
104 kD
B
(Na+ +K+)ATPase
α-subunit expression (AU)
120
90
60
30
0
a
a
b
b
C
S
CE
SE
Experimental groups

Figure 1

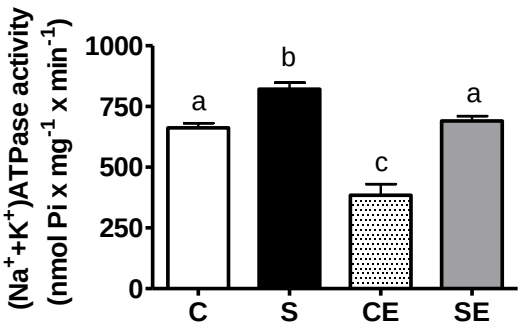
(Na+ +K+)ATPase activity
(nmol Pi x mg-1 x min-1)
1000
750
500
250
0
a
b
c
a
C
S
CE
SE

Figure 2

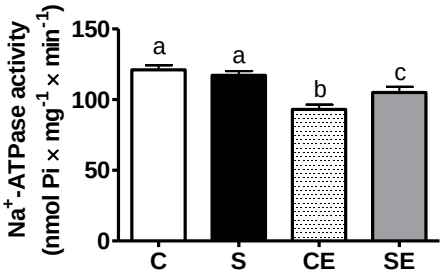

Figure 3

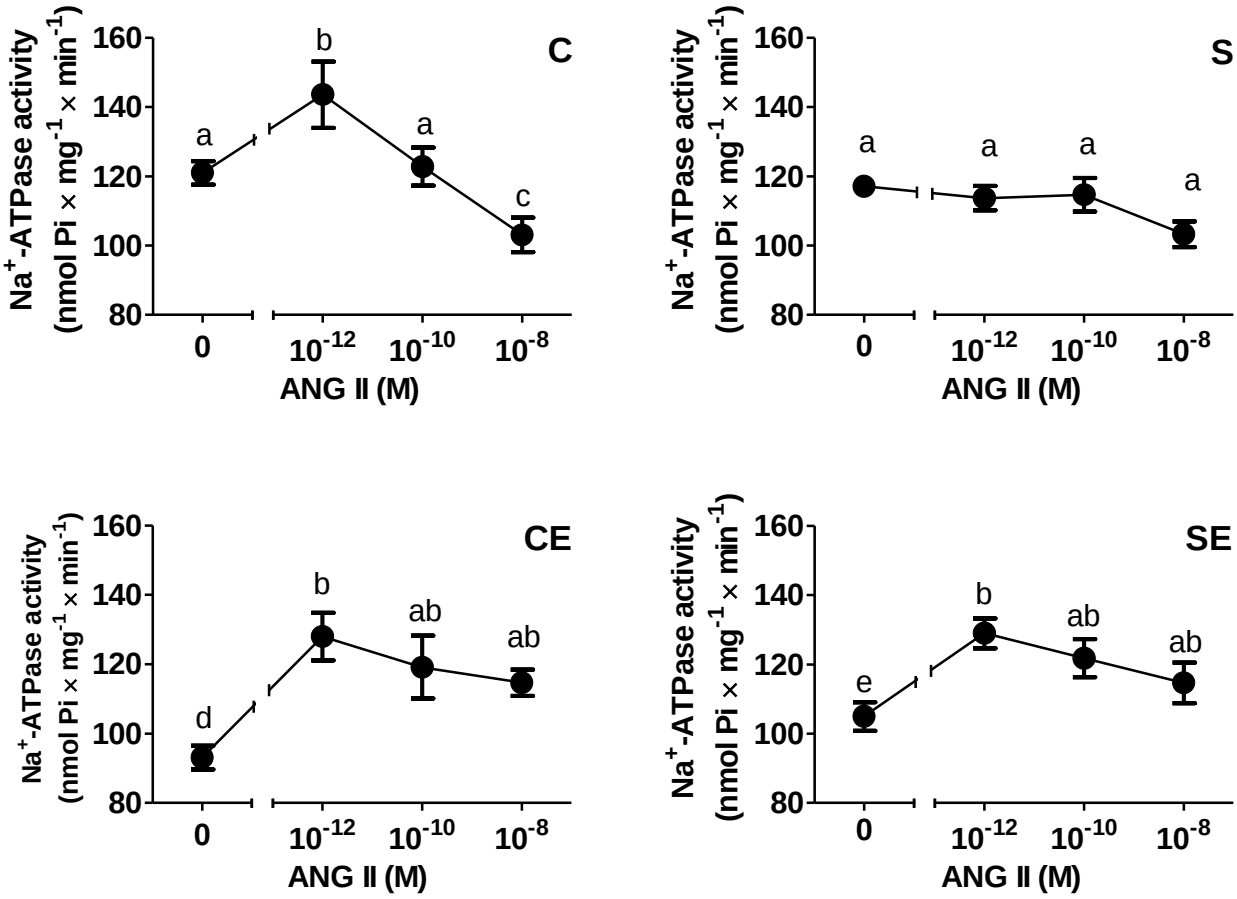
C
S
CE
SE
Na+-ATPase activity
(nmol Pi × mg-1 × min-1)
160
140
120
100
80
a
b
a
c
a
a
a
a
d
b
ab
ab
e
b
ab
ab
0
10-12
10-10
10-8
ANG II (M)

Figure 4

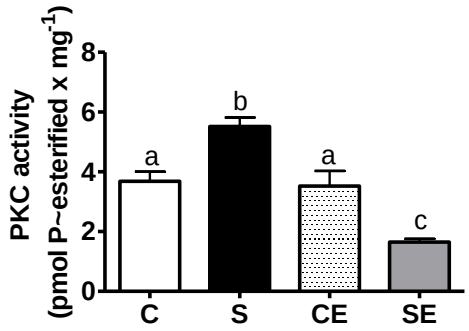

Figure 5

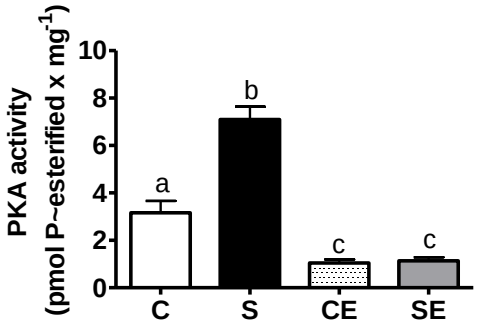

Figure 6

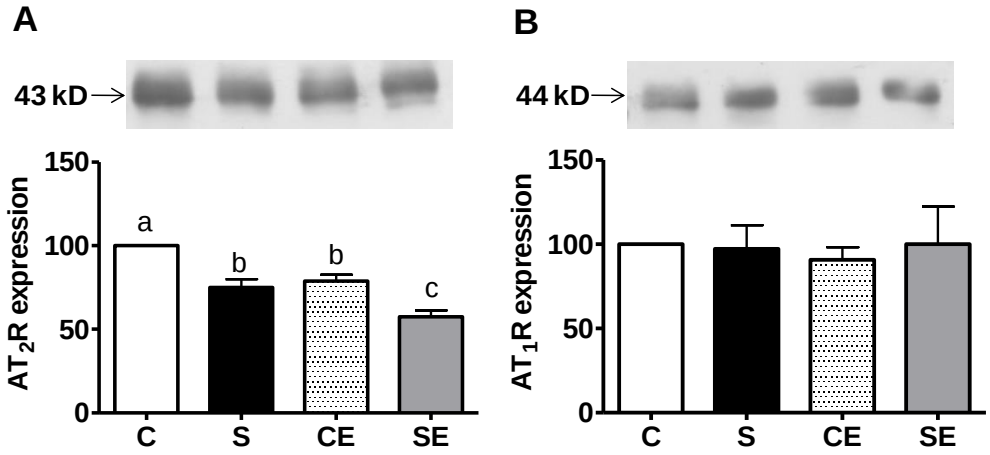
A
43 kD
150
100
50
0
AT2R expression
a
b
b
c
C
S
CE
SE
B
44 kD
150
100
50
0
AT1R expression
C
S
CE
SE

Figure 7

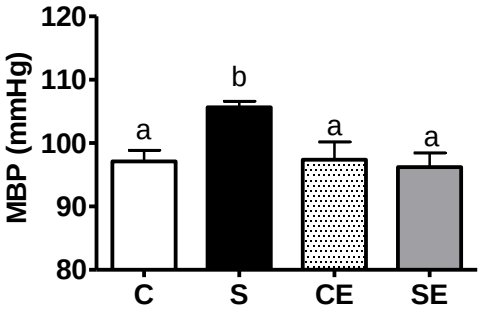

Figure 8

CONCLUSÕES

1. A sobrecarga perinatal de sódio programou alterações moleculares no sistema de sinalização intracelular da ANG II que resultaram em aumento da atividade da bomba $(Na^+ + K^+)$ATPase em membranas do túbulo proximal. Este mecanismo pode ser parcialmente responsável pela hipertensão programada por sobrecarga perinatal de sódio.

2. O tratamento com enalapril, durante curto intervalo de tempo, em período precoce da vida pós-natal, reprograma as alterações moleculares e funcionais produzidas pela sobrecarga de sódio, no entanto leva a programação de baixa densidade de receptores AT_2 que converge com aumento do estresse oxidativo e com outras disfunções renais.

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ANEXO A

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Recife, 24 de abril de 2009

Ofício nº 139/09

Da Comissão de Ética em Experimentação Animal (CEEA) da UFPE
Para: **Profª. Ana Durce Oliveira da Paixão**
Departamento de Fisiologia e Farmacologia
Processo nº 23076.023447/2008 - 34

Os membros da Comissão de Ética em Experimentação Animal do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEEA-UFPE) avaliaram seu projeto de pesquisa intitulado ***"Contribuindo para compreensão de doenças crônicas – degenerativas induzidas por má - nutrição: proliferação mesangial em ratos adultos submetidos a uma sobrecarga de sódio durante a vida intra- uterina".***

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 CEEA-UFPE.

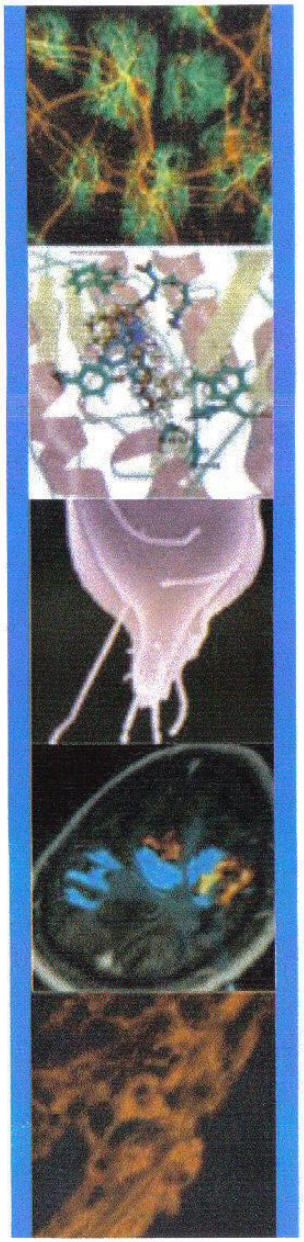
Encontra-se de acordo com as normas vigentes no Brasil, especialmente a Lei 9.605 – art. 32 e Decreto 3.179-art 17, de 21/09/1999, que trata da questão do uso de animais para fins científicos.

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

Atenciosamente,

Profa. Maria Teresa Jansen
Presidente do CEEA
UFPE

ANEXO B



II Encontro Anual do INBEB
2009 – 2010

Instituto Nacional de Ciência e Tecnologia de Biologia Estrutural e Bioimagem

Certificado

Certifico que EDJAIR VICENTE CABRAL apresentou o trabalho "**PERINATAL SALT OVERLOAD IMPRINTS ALTERATIONS IN PROXIMAL TUBULE SODIUM TRANSPORTERS: THESE ALTERATIONS ARE REPROGRAMMED BY SHORT TREATMENT WITH ENALAPRIL AFTER LACTATION**" no II Encontro Anual do INBEB, realizado nos dias 08, 09 e 10 de novembro de 2010, na Universidade Federal do Rio de Janeiro (UFRJ).

Coordenador do INBEB
Professor Jerson Lima da Silva

INBEB
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CAPES