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Implicação do sistema renina angiotensina e da inflamação na hipertensão de ratos programados pela exposição à restrição protéico-calórica na peripuberdade

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Tese apresentada ao Programa de Pós-Graduação em Biociências e Fisiopatologia do Departamento de Análises Clínicas e Biomedicina, Centro de Ciências da Saúde da Universidade Estadual de Maringá, como requisito parcial para obtenção do título de Doutor em Biociências e Fisiopatologia.
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Implicação do sistema renina angiotensina e da inflamação na hipertensão de ratos programados pela exposição à restrição protéico-calórica na peripuberdade

RESUMO

Por décadas, as doenças cardiovasculares têm sido a principal causa de morte no mundo. O mecanismo para essas disfunções pode estar relacionado a disfunções no sistema renina-angiotensina e inflamação. Estudos no contexto do conceito de Origens Desenvolvimentistas da Saúde e da Doença (DOHaD) fornecem evidências de que a exposição a insultos no período peripuberal, como a desnutrição, aumenta a suscetibilidade a doenças crônicas não transmissíveis na idade adulta. No entanto, os mecanismos subjacentes que impulsionam essas disfunções de saúde a longo prazo ainda precisam ser compreendidos. Portanto, o objetivo deste trabalho foi avaliar as implicações a longo prazo do sistema renina-angiotensina e da inflamação na hipertensão induzida por uma restrição proteico-calórica em animais durante o período peripuberal. Ratos *Wistar* machos de trinta dias de idade foram alimentados com uma dieta pobre em proteína (4% de caseína) por 30 dias e, posteriormente, alimentados durante 60 dias com uma dieta controle com 20,5% de proteína para uma recuperação alimentar (grupo LP). Os animais do grupo controle (grupo NP) foram alimentados com uma dieta controle com 20,5% de proteína durante todo o período. Os parâmetros cardiovasculares, inflamatórios, e o sistema renina-angiotensina foram avaliadas no dia 120 após o nascimento. Os animais LP apresentam aumento da pressão arterial. A curva dose-resposta da angiotensina 2 dos animais LP mostrou um aumento na resposta pressórica em uma dose menor (50 ng/kg). No coração e na aorta, os níveis de mRNA do receptor AT1 foram aumentados; no entanto, os níveis de mRNA do receptor AT2 foram reduzidos no coração e aumentados na aorta. Em outra coorte de animais, o ecocardiograma mostrou que o grupo LP apresentou um aumento na espessura do septo interventricular, diâmetros internos, espessura do ventrículo esquerdo e fração de encurtamento. No eletrocardiograma, os ratos LP também mostraram um aumento tanto da amplitude da onda T como do índice cárdio simpático aumentado. A expressão de RNAm mostrou um aumento do TNF-α e IL6 no coração. No encéfalo, os animais LP mostraram um número aumentado de células positivas para Iba-1 no núcleo paraventricular do hipotálamo, no núcleo intermediário do trato solitário (iNTS) e na medula ventrolateral rostral. Sugere-se que a inflamação e a disfunção do sistema renina-angiotensina podem mediar a hipertensão neurogênica adulta e a disfunção cardíaca induzida pela restrição proteica peripuberal.

Palavras-chave: Desnutrição. Disfunção cardiovascular. Programação cardiometabólica. Inflamação cardíaca. Angiotensina 2.

Implication of the renin-angiotensin system and inflammation in hypertension of rats programmed by caloric-protein restriction exposure in peripuberty

ABSTRACT

For decades, cardiovascular disease has been the leading cause of death in the world. The mechanism for these dysfunctions can be related to dysfunctions in the renin-angiotensin system and inflammation. Studies in the context of the Developmental Origins of Health and Disease (DOHaD) concept provide evidence that exposure to early life insults in the peripubertal period, such as malnutrition increases susceptibility to noncommunicable chronic diseases in adulthood. However, the underlying mechanisms driving these long-term health dysfunctions remain to be fully understood. Therefore, the aim of this work was to evaluate the long-term implications of the renin-angiotensin system and inflammation in hypertension induced by protein-calorie restriction in animals during the peripubertal period. Thirty-day-old male Wistar rats were fed a low-protein diet (4 % casein) for 30 days and subsequently fed with a control diet for 60-days with a normal protein diet with 20.5 % for a dietary recovery (LP group). Control animals (NP group) were fed with a control diet with 20.5 % protein diet throughout their lives. Cardiovascular, inflammatory parameters and the renin-angiotensin system were evaluated on postnatal day 120. LP animals show increase in blood pressure. The angiotensin 2 dose-response curve of LP animals showed an increase in the pressor response at a lower dose (50 ng/kg). In the heart and aorta, AT1 receptor mRNA levels were increased; however, the AT2 receptor mRNA levels were reduced in the heart and increased in the aorta. In another cohort of animals in the echocardiogram, the LP group showed an increase in the thickness of the interventricular septum, internal diameters, thickness of the left ventricle, and the shortening fraction. In the electrocardiogram, LP rats also showed an increase in both T wave amplitude and increased cardiac sympathetic index. mRNA expression showed an increase in TNF-α and IL6 in the heart. In the brain, LP animals showed an increased number of Iba-1 positive cells in the paraventricular nucleus of the hypothalamus, the intermediate nucleus of the solitary tract (iNTS), and the rostral ventrolateral medulla. It is suggested that inflammation and renin-angiotensin system dysfunction may mediate adult neurogenic hypertension and cardiac dysfunction induced by peripubertal protein restriction.

Keywords: Malnutrition. Cardiovascular dysfunction. Cardiometabolic program. Cardiac inflammation. Angiotensin 2.

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Sumário

CAPÍTULO I

Introdução

As doenças cardiovasculares são a principal causa de morte no mundo, afetando mais de meio bilhão de pessoas globalmente. Em 2021, estima-se que 20,5 milhões de pessoas morreram em decorrência dessas doenças, na qual, representa quase um terço de todas as mortes do mundo. (1). A presença de fatores de risco como a hipertensão, diabetes, dislipidemia podem aumentar o risco cardiovascular (2). Outros estudos demonstram que o consumo de dietas inadequadas (dietas com baixa proteína ou rica em gordura) podem contribuir para o aumento da incidência de disfunções cardiometabólicas (3,4). Paradoxalmente a desnutrição tem se tornado um problema de saúde pública, estimando-se que em 2023, 733 milhões de pessoas enfrentam a desnutrição, sendo a sua maior prevalência na África, Asia, América Latina e Caribe com repercussões no desenvolvimento econômico, social e na saúde (5).

Sugere-se que a desnutrição é um dos principais fatores de risco para as doenças cardiovasculares. Estudos sobre o conceito de *Developmental Origins of Health and Disease* (DOHaD) demonstram que insultos como a desnutrição, quando realizados em fases críticas do desenvolvimento, são um dos principais preditores de doenças cardiovasculares na vida adulta (3,6–8). Dentre os mecanismos para essas disfunções, podemos destacar a contribuição do sistema nervoso autonômico (3), sistema renina angiotensina (7) e da inflamação (9,10).

Alguns modelos de hipertensão apresentam um forte componente autonômico, dessa forma esse quadro de hipertensão se enquadra na classificação de hipertensão neurogênica (11). Nesse modelo de hipertensão ocorre a elevação crônica do sistema nervoso simpático, que aumenta o fluxo simpático para o coração e vasos. Associado ao aumento do tônus simpático, temos duas características que são primárias da hipertensão neurogênica, a desregulação dos componentes do sistema renina angiotensina (12) e uma neuroinflamação (13).

Developmental Origins of Health and Disease (DOHaD)

Estudos publicados desde 1930 demonstram relação entre as condições ambientais durante a vida fetal e a infância pareciam estar determinando o estado de saúde na vida adulta (14). Quando esses insultos ocorrem em períodos de grande plasticidade dos órgãos e sistemas podem desencadear efeitos duradouros no indivíduo, alterando a função e/ou estrutura de

órgãos, programando para o surgimento doenças na vida adulta (15). Na década de 1970, Ravelli e colaboradores realizaram um estudo com 300.000 homens, filhos de mulheres expostas a um período de escassez alimentar (fome holandesa), durante a Segunda Guerra Mundial (16). Na vida adulta, eles apresentaram padrões diferenciados de composição corporal dependendo da idade em que tinham sido expostos à desnutrição materna durante a vida intrauterina. Quando a mãe foi exposta a desnutrição durante o último trimestre da gestação, esse grupo demonstrou uma baixa incidência de obesidade. Quando a desnutrição ocorreu no primeiro semestre da gestação, a incidência de obesidade aumentou significativamente na prole (16).

Dentre os insultos de malnutrição abordados na literatura, podemos destacar os insultos com dieta, tanto hipercalórica como com baixa proteína. As principais fases susceptíveis para a programação de doenças na vida adulta são o período prévio a concepção, a gestação, a lactação, a infância e a peripuberdade (17,18).

Diversos estudos na literatura também enfatizam a gestação e/ou lactação como fases críticas do desenvolvimento (6,8,19). Alicerçados ao modelo de repostas preditivas adaptativas, estudos que exploram a fase perinatal sugerem que durante o desenvolvimento sinais hormonais transmitidos pela mãe através da placenta e/ou leite materno desencadeiam um ajuste no funcionamento dos órgãos e sistemas do bebê permitindo-lhe "prever" e estar preparado para o ambiente em que estará inserido futuramente (20). Considerando que a previsão do ambiente externo está correta, o risco para doenças é considerado baixo (match). No entanto, se o ambiente pós lactacional é diferente do previsto no ambiente de alta plasticidade (gestação e/ou lactação), ocorre um maior risco para doenças (mismatch) (21), programando para alterações epigenéticas com efeitos transgeracionais (22). Estudos demonstram que insultos como a abundância nutricional (com dieta rica em gordura) ou restrição proteica na fase perinatal podem programar para disfunções cardiometabólicas na vida adulta (4,6,23,24); evidenciando disfunções no metabolismo da glicose, aumento da pressão arterial, aumento do tônus simpático vascular e hipertrofia do ventrículo esquerdo na vida adulta (4,6,8,25).

A peripuberdade é considerada uma das últimas, se não a última, fase crítica do desenvolvimento susceptível aos insultos que podem programar para disfunções cardiometabólicas na vida adulta (26). Biologicamente, nessa fase ocorrem modificações a nível neural e endócrino durante que resulta em alterações morfológicas no corpo, finalização do amadurecimento do córtex pré-frontal e outras áreas do sistema nervoso cental, maturação das características sexuais primárias e secundárias e pôr fim a aquisição da maturidade

reprodutiva e neuronal (27–29). É importante ressaltar que nessa fase, pode ocorrer maior exposição a fatores estressores ambientais e sociais devido ao processo de amadurecimento e demandas sociais (30), com maior exposição a dietas inadequadas, com pouca quantidade de proteína e alto valor calórico (31–33).

Evidenciando que a peripuberdade é uma fase crítica do desenvolvimento, estudos do nosso grupo de pesquisa, demonstraram que a exposição ao consumo de água rica em sal (0.3 M NaCl) na peripuberdade, programa para hipertensão arterial, diminuição da sensibilidade do barorreflexo, hipertrofia de cardiomiócitos com um aumento da fibrose perivascular e intersticial no coração aos 120 dias (34). Também demonstramos que os ratos expostos a dieta rica em gordura (35% de gordura) foram programados para uma disfunção cardiometabólica na vida adulta com aumento de adiposidades, disfunção no metabolismo da glicose, além de alterações no sistema reprodutor evidenciado pelo aumento anormal dos túbulos seminíferos, redução na altura do epitélio e no diâmetro dos túbulos seminíferos (23,35). Mais recentemente, nosso grupo demonstrou que a restrição proteica na peripuberdade promove hipertensão com disfunções cardiometabólicas, reprodutivas e endoteliais na vida adulta (3,7,36–38).

Desnutrição

A desnutrição conforme definida pela Organização Mundial de Saúde (OMS), refere-se a deficiências ou excessos na ingestão de nutrientes, desequilíbrio de nutrientes essenciais ou utilização prejudicada de nutrientes(39). A OMS em parceria com outras organizações de saúde, publicou o relatório de segurança alimentar e nutricional, acompanhando o estado de desnutrição e a fome no mundo. Na edição publicada em 2024, foi reportado um aumento da desnutrição no mundo desde 2019, devido a pandemia de COVID-19 (5). Em 2023 o número de pessoas que enfrentou a fome continuou sendo maior na Ásia (384.5 milhões), estando a África em segundo lugar com (294 milhões), seguida pela América Latina e o Caribe (41 milhões). Projeções atualizadas apontam que 582 milhões de pessoas estarão cronicamente subnutridas em 2030, apontando para o imenso desafio de alcançar o ODS 2 (Objetivos do Desenvolvimento Sustentável - Fome Zero) tornando-se, portanto, esta, uma questão de saúde pública mundial (5).

A volta do Brasil ao mapa da fome foi uma das consequências das reduções de políticas públicas promovidas ao longo dos anos (40) e em uma pesquisa realizada em 2021-2022 pela Rede Brasileira de Pesquisa em Soberania e Segurança Alimentar e Nutricional (Rede

PENSSAN), demonstrou um aumento da fome no Brasil nesse período, pois 33,1 milhões de Brasileiros não conseguiam ter as suas necessidades alimentares básicas atendidas e seis em cada dez brasileiros (58,7% da população) conviveram com algum grau de insegurança alimentar (41).

Um dos principais fatores que podem agravar a insegurança alimentar, é o alto custo dos alimentos mais nutritivos, tendo como consequência a falta de acessibilidade das dietas saudáveis para as famílias. Estima-se que 3 bilhões de pessoas no mundo não consigam pagar por uma alimentação saudável, pois os alimentos mais ricos em nutrientes como os laticínios, frutas, vegetais e alimentos ricos em proteínas são o grupo de alimentos mais caros do mundo (5). Desta forma, há maior predisposição para o consumo de café, arroz, margarina, feijão, farinha e pão, com uma maior preferência por açúcares e *fast foods*. Tendo como consequência o consumo de uma alimentação pobre em nutrientes e pro-inflamatória (42,43).

Desnutrição na fase da peripuberdade

A peripuberdade é considerada uma fase crítica do desenvolvimento ainda pouco explorada na literatura no contexto da programação cardiometabólica induzida por um modelo de restrição proteica. Nosso grupo de pesquisa tem explorado este modelo nos últimos 12 anos resultando na publicação de seis artigos que avaliaram parâmetros cardiometabólicos, endoteliais e reprodutivos (3,7,36–38,44).

Nos primeiros dois estudos avaliamos os parâmetros metabólicos e demonstramos que esses animais que passaram pela restrição proteica na peripuberdade foram programados para disfunções do metabolismo na vida adulta (38,44). Mostramos que estes animais apresentam hiperglicemia, hiperleptinemia, hiperinsulinemia e dislipidemia, aumento dos estoques de gordura, hipersecreção de insulina e disfunção autonômica (38,44).

Quando avaliados os parâmetros reprodutivos desses animais desnutridos, observamos que após o insulto na peripuberdade esses animais apresentaram testículos significativamente menores, com redução na quantidade de células germinativas e nas células de Sertoli, essenciais para a produção de espermatozoides (36). Eles também apresentam uma diminuição na maturação dos espermatozoides no epidídimo, com alterações morfológicas que podem comprometer a capacidade de armazenamento e maturação dos espermatozoides. Na avaliação dos espermatozoides, encontramos uma redução na motilidade e alterações morfológicas, na qual, sugere um comprometimento na capacidade de fertilização. Mesmo após a normalização com a dieta padrão, parte as alterações observadas na peripuberdade persistiram na vida adulta.

Evidenciado por testículos menores e alterações epididimárias. Nos espermatozoides, ocorreu uma diminuição na motilidade e a presença de espermatozoides morfologicamente anormais, indicando comprometimento a longo prazo da capacidade de reprodução (36).

Quando avaliada a função endotelial da aorta, demonstramos que os animais LP apresentam, na vida adulta, um aumento da resposta a fenilefrina em anéis aórticos com endotélio. Esse aumento da contratilidade da aorta, pode estar relacionado ao aumento da produção do ânion superóxido pela enzina NADPH oxidase, considerando que quando as aortas foram incubadas com apocinina observou-se a recuperação da modulação endotelial. Em adição a esses resultados também encontramos um aumento da peroxidação lipídica e redução da catalase nas aortas dos animais LP, evidenciando que a disfunção endotelial pode estar sendo mediada pelos mecanismos de estresse oxidativo (37).

Concomitante com esses trabalhos, durante o desenvolvimento do mestrado foi verificado que esses animais apresentam disfunção cardiovascular com uma hipertensão neurogênica. Eles apresentam aumento do tônus simpático cardíaco e do diâmetro dos cardiomiócitos e uma maior deposição de colágeno no coração. Avaliamos também o tônus simpático vascular nesses animais e foi evidenciado um aumento da resposta ao hexametônio, nos animais desnutridos (3).

Sabe-se que um importante modulador da atividade simpática é a angiotensina 2, promovendo tanto vasoconstrição direta, que ativa o sistema nervoso simpático e estimula a liberação de norepinefrina nas terminações nervosas simpáticas; ou aumenta a liberação de adrenalina da medula adrenal, que resulta em aumento da frequência cardíaca e da força de contração do coração (45). Concomitante com esse mecanismo, a ativação simpática pode resultar na ativação do SRA resultando na produção de mais angiotensina 2 (46). Além do mais, a liberação de norepinefrina pelos nervos simpáticos resulta na liberação de renina pelos rins, dando início a cascata que resulta na formação de angiotensina 2 (46).

Neste contexto, durante este doutorado foi investigado como o sistema renina angiotensina poderia contribuir para a hipertensão neurogênica encontrada nos animais desnutridos. Os dados deste estudo resultaram no primeiro artigo publicado desta tese onde ficou evidenciado que os animais LP apresentam um aumento da angiotensina 2 circulante, com uma maior resposta pressórica a angiotensina 2 (7). Quando avaliado o SRA no coração e aorta, foi verificado um aumento do RNA mensageiro para o receptor AT1, ECA1 com uma redução da ECA2 e MAS no coração e aorta (7).

Outro mecanismo que poderia estar implicado na hipertensão dos animais LP seria a inflamação tanto periférica quanto central. No segundo artigo da tese (dados não publicados)

foi explorado o papel da inflamação nos animais desnutridos. Observa-se que esses animais apresentam um aumento do RNA mensageiro para IL6 e TNF alfa no coração com um aumento da neuroinflamação no núcleo paraventricular do hipotálamo (PVN) e o bulbo rostroventrolateral (RVLM) e núcleo do trato solitário (cNTS e iNTS). Aparentemente tais alterações podem estar mediando a hiperatividade simpática e disfunção cardíaca observadas neste modelo e que serão melhor exploradas nos tópicos seguintes.

Considerando que no primeiro artigo desta tese ficou evidente um aumento no sistema renina angiotensina nos animais LP (7). O aumento na angiotensina 2 circulante pode estar induzindo a ativação de áreas de controle cardiovascular responsivas ao estímulo do CVOs, resultando na geração e transmissão de sinais hipertensivos para o PVN (13). A micróglia no PVN é ativada com um aumento nas citocinas pró-inflamatórias, aumentando direta ou indiretamente a atividade dos neurônios simpáticos do PVN (13). Essas e outras vias convergem para o RVLM, levando a aumentos no fluxo simpático (13,47), aumentando assim a pressão arterial.

Remodelamento cardíaco

O remodelamento cardíaco é definido como um conjunto de alterações moleculares, celulares e intersticiais que se manifestam clinicamente como alterações no tamanho, forma e função do coração resultantes de lesão cardíaca (48). Para o diagnóstico da remodelação cardíaca patológico é baseado na detecção de alterações morfológicas, como por exemplo as alterações no diâmetro da cavidade, massa (hipertrofia e atrofia), geometria (espessura e forma da parede cardíaca), áreas de cicatriz após infarto, fibrose e infiltrado inflamatório (48).

A disfunção cardíaca é a principal consequência da remodelação cardíaca, que contribui para o surgimento e progressão da disfunção ventricular. Essa interação começa com alterações genéticas a lesão cardíaca, com re-expressão de genes fetais. Tendo como consequência alterações celulares e moleculares, resultando em perda progressiva da função ventricular, a qual pode evoluir para insuficiência cardíaca(49).

Nos estudos com modelos de programação com animais LP durante a gestação encontramos um perfil de remodelamento cardíaco com um quadro mais avançado de desorganização morfológica cardíaca aos 60 ou 112 dias de vida, pois observa-se uma deposição de colágeno no coração e diminuição do número de cardiomiócitos (8,50), além de

modificações da expressão de microRNAs no coração relacionados a estrutura e função cardíaca (8).

Os animais LP que passaram pelo insulto de restrição proteica na peripuberdade, logo após o insulto, aos 60 dias, eles demonstram um aumento da massa cardíaca com um aumento da fração de encurtamento e da parede posterior do ventrículo esquerdo no ecocardiograma (51). Quando foi avaliado esses animais na vida adulta, demonstraram um aumento do diâmetro dos cardiomiócitos e da fibrose intersticial (3). Durante o doutorado a continuação deste estudo demonstrou que o coração dos animais LP apresenta um aumento do diâmetro interno e da parede posterior do ventrículo esquerdo com uma maior fração de encurtamento. Quando avaliada a inflamação cardíaca, os animais LP demonstraram um aumento do mRNA de IL6 e TNF alfa no coração.

Na literatura, não foi encontrado artigos que realizam a desnutrição proteica durante todo o período da peripuberdade que realizam a avaliação dos parâmetros inflamatórios após o insulto ou na vida adulta. No entanto, um estudo que realizou restrição proteica (2% de proteína) no final da peripuberdade (~56 dias) demonstrou que esses animais apresentaram um aumento da inflamação sistêmica, com elevação dos níveis de TNF-α, IL-6 e IL-1β circulantes aos 70 dias (52). Essa inflamação crônica de baixo grau também pode ser observada quando a restrição proteica é realizada durante a gestação e lactação, resultando em aumento do TNF-α e uma tendência de elevação do IL-6 circulante (10), na qual, pode contribuir para o desenvolvimento de doenças cardíacas. Vários outros estudos também demonstraram que a inflamação está relacionada a hipertensão, hipertrofia, fibrose e remodelamento cardíaco (53–55).

Síndrome cardiometabólica

A síndrome cardiometabólica é caracterizada pelo conjunto de condições clínicas que aumentam a chance de desenvolver doenças cardiovasculares, acidente vascular cerebral e diabetes tipo 2 tornando-se uma das principais causas de mortalidade e morbidade no mundo (56). É caracterizada pela presença de três ou mais fatores de risco, como acúmulo de gordura abdominal, dislipidemia (alterações no nível de colesterol ou triglicerídeos), hipertensão, hiperglicemia e resistência à insulina resultando na síndrome cardiometabólica (57). A inflamação crônica de baixo grau, caracterizada por níveis elevados de marcadores inflamatórios, desempenha um papel central na patogênese da síndrome cardiometabólica (57). No modelo de desnutrição na peripuberdade em estudos prévios do nosso laboratório demonstramos que esses animais possuem um maior acúmulo de gordura visceral, aumento dos

triglicerídeos com um aumento da pressão arterial na vida adulta. Foi evidenciado também hiperglicemia e resistência à insulina (7,38).

A desorganização do tecido adiposo pode contribuir para eventos de apoptose e necrose (58). Com o aumento progressivo dos adipócitos, ocorre também a infiltração de macrófagos, que realizam a produção de citocinas pró inflamatórias, como o fator de necrose tumoral-alfa, produção de espécies reativas de oxigênio, que podem estimular a inflamação e comprometem a função do tecido adiposo (59). Esses fatores estão relacionados à inflamação e a diminuição da sinalização da insulina contribuindo, assim, para o desenvolvimento da resistência à insulina (60).

A pressão arterial é a força que o sangue exerce sobre as paredes das artérias. Na hipertensão ocorre a elevação persistente da pressão arterial, caracterizada por valores de pressão arterial sistólica de >140 mmHg ou mais e/ou pressão arterial diastólica mais de >90 mmHg (61). A hipertensão é uma doença multifatorial que pode resultar em alterações neuronais, hormonais, vasculares e renais, além de estar associada a disfunções metabólicas (62). A presença de fatores de risco como dieta desequilibrada, sedentarismo, idade, obesidade, inflamação podem desencadear ou agravar a hipertensão (63,64). A origem da hipertensão pode estar relacionada a mecanismos de regulação rápida da pressão arterial, como o sistema nervoso autonômico, bem como a mecanismos de regulação a longo prazo, mediada pelos rins através do sistema renina angiotensina aldosterona (65).

A resistência à insulina, é uma das principais condições clínicas que podem acompanhar a instalação da síndrome cardiometabólica. A resistência à insulina é caracterizada pela redução da eficácia da insulina em promover a captação de glicose pelas células, mesmo em concentrações plasmáticas normais. Resultando em uma elevação compensatória nos níveis de insulina (hiperinsulinemia), na tentativa de manter os níveis de glicose sanguínea dentro dos limites normais (66). Nesse contexto, a insulina é reconhecida como um hormônio metabólico, produzido pelas células β do pâncreas, que estimula a captação de glicose em diversos órgãos, especialmente nos músculos, tecido adiposo e fígado, além disso, a insulina exerce um efeito inibidor sobre a lipólise no tecido adiposo (67).

Distúrbios na produção e/ou na sinalização da via da insulina e a resistência dos tecidos frequentemente estão ligados a estágios iniciais de níveis elevados de glicose no sangue (68). Na literatura, a RI acompanhada de hiperinsulinemia e hiperglicemia é sugerida como um dos mecanismos unificadores para doenças relacionadas à síndrome cardiometabólica (69). O

acúmulo de gordura na região abdominal é considerado um determinante crítico da RI em todo o corpo, considerando que no tecido adiposo, a insulina estimula processos como o metabolismo e a captação de glicose, o armazenamento de gordura no processo de lipogênese e o transporte de ácidos graxos da corrente sanguínea para as células (68).

A resistência à insulina está fortemente associada à meta-inflamação, que é caracterizada por um estado de inflamação crônica de baixo grau, frequentemente observado nas doenças cardiometabólicas e na obesidade (70). Esse perfil de resistência à insulina, resulta na ativação do sistema imunológico, principalmente dos macrófagos, que podem infiltrar o tecido adiposo (71). Como consequência, há a liberação de citocinas pró-inflamatórias, como TNF-α, IL-6 e IL-1β, na qual podem exacerbar a resistência à insulina interferindo na sinalização de insulina e promovendo uma inflamação sistêmica (72). A meta-inflamação, portanto, pode resultar em um ciclo patológico, no qual a inflamação crônica contribui para o desenvolvimento de disfunções cardiometabólicas (73).

Hipertensão

A prevalência da hipertensão (valores de pressão arterial superiores 140/90 mmHg) dobrou entre 1990 e 2019, passando de 650 milhões para 1,3 bilhão, sendo a maior incidência observada em países de baixa e média renda (74). O estilo de vida possui um profundo impacto no aumento da incidência de pessoas com hipertensão, considerando que a implementação de hábitos saudáveis, como atividade física regular, alimentação equilibrada, manejo do estresse, cessação do tabagismo e sono adequado, pode reduzir significativamente o risco de doenças cardiovasculares e promover uma melhora no bem-estar cardiovascular geral (75). Pacientes hipertensos podem ser assintomáticos; no entanto, quando os sintomas se manifestam, incluem cefaleias matinais, dor torácica e alterações na visão (61). Em casos mais graves (valores de pressão arterial superiores a 180/120 mmHg), podem ocorrer cefaleia intensa, dor torácica, vertigem, dispneia, náusea, distúrbios visuais, ansiedade, confusão mental, e arritmia cardíaca (61).

A hipertensão clínica pode ser classificada em duas categorias amplas. A hipertensão primária (ou essencial) na qual ocorre em 85% e 95% e possui uma causa não identificada, podendo a sua origem geralmente estar relacionada a interação de vários fatores como a inflamação, estresse oxidativo, com alterações da função renal, neural e vascular (62). Para o

tratamento, são usadas medidas não farmacológicas, relacionadas à modificação do estilo de vida, como mudanças na dieta, prática de atividade física, redução do consumo de cafeína, álcool e tabaco, associadas ao tratamento farmacológico (76). A hipertensão secundária é causada por condições subjacentes identificáveis, incluindo aldosteronismo primário, doença renovascular, doença renal crônica, apneia obstrutiva do sono, tumores secretores de catecolaminas (feocromocitoma), coarctação da aorta e hipertensão induzida por drogas ou álcool (77). A hipertensão neurogênica também é considerada uma forma de hipertensão secundaria resultante do aumento da atividade simpática para o sistema cardiovascular, que desencadeia em um aumento de pressão arterial pode estar associado a uma maior sobrecarga cardíaca estrutural e funcional (78).

Regulação central da pressão arterial

A neuroinflamação no tronco encefálico nos núcleos de regulação cardiovascular tem sido descrita como um dos importantes mediadores da hipertensão (79). Considera-se que o núcleo paraventricular do hipotálamo (PVN), bulbo ventrolateral rostral (RVLM) e o núcleo do trato solitário (NTS) atuam no controle cardiovascular, mediando a atividade do sistema nervoso autonômico, incluindo o tônus simpático cardiovascular (80).

O NTS integra sinais aferentes advindos dos barorreceptores cardiopulmonares e quimiorreceptores, realiza também o envio das projeções glutamatérgicas para os interneurônios nitrérgicos e gabaérgicos ao redor do PVN, assim como para os neurônios pré-sinápticos no PVN (79,81,82). O NTS também possui conexões excitatórias diretas com a coluna intermediolateral (IML) e conexões inibitórias com o RVLM através das projeções glutamatergicas para neurônios gabaérgicos da medula ventrolateral caudal (CVLM) (81).

Os neurônios parvocelulares pré-autonômicos do PVN contribuem para o controle do sistema nervoso simpático e estão implicados na simpatoexicatação relacionada a hipertensão e disfunção cardíaca(83). O PVN possui ramificações para o RVLM e para o NTS, enviando projeções descendentes para a IML. A atividade desses neurônios é modulada por interneurônios gabaérgicos e nitrérgicos ao redor do PVN (84).

O RVLM é considerado uma das áreas mais importantes para o controle simpático cardiovascular, dessa forma quando esses neurônios são ativados ocorre uma maior estimulação simpática para os vasos sanguíneos e coração, promovendo vasoconstrição, aumento da frequência cardíaca e força de contração do coração(85). O RVLM possui neurônios simpáticos

pré-ganglionares que se projetam para a coluna intermediolateral (IML) na região toracolombar da medula espinhal, onde realiza sinapses com neurônios simpáticos pós-ganglionares em regiões pré e paravertebrais e na medula da adrenal. Os neurônios do RVLM também recebem projeções glutamatergicas do PVN e gabaérgicas do CVLM (79).

Sistema Renina Angiotensina

No contexto experimental, vários modelos animais de hipertensão, como por exemplo: o modelo de hipertensão essencial (ou primária), o rato espontaneamente hipertenso (SHR), o modelo de hipertensão secundária (hipertensão renovascular), ou animais obesos com hipertensão; apresentam aumento da atividade simpática, aumento da atividade do sistema renina-angiotensina (inclusive no sistema nervoso central (SNC)) e aumento inflamação de baixo grau (metainflammação) (86–88).

O sistema renina angiotensina (SRA) auxilia no controle da pressão arterial e do equilíbrio eletrolítico. A angiotensina 2 (Ang 2) é o principal efetor do SRA. Ela é produzida pela reação em cascata enzimática onde a renina é a enzima chave que cliva o angiotensinogênio produzido no fígado gerando angiotensina 1 (85). A enzima conversora de angiotensina (ECA) cliva a angiotensina 1 produzindo a angiotensina 2 que se liga ao receptor AT1. A ativação do receptor AT1 promove maior atividade vasoconstritora e aumenta o volume sanguíneo através da aldosterona e contribui para o aumento da atividade simpática (85).

No início da cascata de ativação do SRA, temos a renina. Para a sua formação, nas células justaglomerulares que estão localizadas nas arteríolas aferentes do rim, possuem a prorenina. A ativação dessas células, causa a clivagem da prorenina em renina. Pode ser liberada na circulação por alguns estímulos, como por exemplo quando ocorre alterações na perfusão renal; redução no fluxo sanguíneo, quando o túbulo contorcido distal recebe menos sódio e cloreto. Essas alterações são percebidas pela mácula densa, que então ativa um mecanismo de feedback. A angiotensina 1, é parte desse feedback, ajudando a regular a pressão e o fluxo sanguíneo. Além disso, o potássio e o ANP (peptídeo natriurético atrial) também desempenham papéis importantes nesse processo, ajudando a equilibrar o volume de sódio, água e a pressão arterial nos rins (89,90). O angiotensinogênio é produzido é secretado principalmente pelo fígado. A renina cliva a porção N-terminal do angiotensinogênio resultando na formação da angiotensina 1 (89).

A enzima conversora de angiotensina (ECA) é expressa na membrana plasmática das células endoteliais vasculares principalmente na circulação pulmonar, realizando a clivagem de

2 aminoácidos da porção C-terminal da angiotensina 1 para formar a angiotensina 2 (91). A ECA também age na via das bradicininas, que possuem efeitos de redução da pressão arterial, e realiza a hidrólise da bradicinina, inativando-a (91). Dessa forma a ECA pode aumentar a produção de um vasoconstritor (angiotensina 2) e diminui a disponibilidade de um vasodilatador (bradicinina). Os fármacos que realizam a inibição da ECA, reduzem tanto a vasoconstrição induzida pela angiotensina 2 como o bloqueio da degradação da bradicinina (92). Esses fármacos podem prevenir a hipertrofia cardíaca e facilitam a excreção de sal e água (91).

A angiotensina 2 é o principal mediador dos efeitos do SRA, com efeitos na regulação da pressão arterial, regulação do volume e secreção de aldosterona (93). Os efeitos da angiotensina 2 para regulação da pressão arterial e do volume podem ser mediados pela vasoconstrição através da contração do musculo liso vascular nas arteríolas (94); secreção de aldosterona do córtex adrenal (94); aumento da atividade simpática do sistema nervoso central (95) e aumento da liberação de vasopressina pelo hipotálamo (96).

A angiotensina 2 também está implicada em alterações fisiopatológica, relacionadas ao aumento do estresse oxidativo, vasoconstrição, disfunção endotelial, fibrose e hipertrofia cardíaca (97). Considerando esses efeitos a angiotensina 2 está implicada na hipertensão, aterosclerose, insuficiência cardíaca e doença renal (98). Os efeitos da angiotensina 2 podem ser mediados por dois tipos de receptores, os do tipo 1 e do tipo 2.

O receptor do tipo 1 (AT1) é um receptor acoplado a proteína G, que pode ser encontrado no coração, vaso, rim, glândulas suprarrenais, hipófise e sistema nervoso central. Após a ligação da angiotensina 2 com receptor AT1, foi demonstrado que esse receptor expresso no sistema cardiovascular ativa várias proteínas cinase intracelulares(99). Estudos demonstram que essas cinases estimulem a NADPH oxidase, a produção de espécies reativas de oxigênio e a síntese de proteínas, resultando na hipertrofia, hiperplasia e hipertrofia cardíaca (99).

O receptor do tipo 2 (AT2) também é acoplado a proteína G expresso principalmente no coração, cérebro, rins e glândulas adrenais (100). O receptor AT2 realiza efeitos opostos e protetores da angiotensina 2 via receptor AT1. Inibindo a inflamação, a fibrose e o fluxo simpático central e causando vasodilatação (101).

A angiotensina 2 também pode exercer seus efeitos inflamatórios principalmente pela ativação de receptores específicos, como o AT1, presentes em várias células do sistema imune, como macrófagos e linfócitos (102). Quando a angiotensina 2 se liga a esses receptores, resulta na liberação de citocinas pró-inflamatórias, que promovem uma resposta inflamatória local e

sistêmica (103). Além disso, a angiotensina 2 pode aumentar a expressão de moléculas de adesão nas células endoteliais, facilitando a migração de leucócitos para os tecidos inflamatórios (102). Outro mecanismo importante é a indução de estresse oxidativo, que gera espécies reativas de oxigênio, contribuindo para a ativação de vias de sinalização inflamatórias. Dessa forma, a angiotensina 2 está envolvida tanto na regulação da pressão arterial, como no aumento da inflamação, exacerbando disfunções cardíacas (104).

Neuroinflamação

Na literatura estudos demonstram que as micróglias podem ser ativadas na hipertensão, especialmente se essas células forem encontradas nos núcleos cardiovascular de controle da pressão arterial (PVN, NTS e RVLM), resultando em hiperatividade simpática periférica (105) e remodelação miocárdica patológica (106). As micróglias são as principais células neuroinflamatórias do SNC, na qual detectam padrões moleculares associados a danos (DAMPs) ou padrões moleculares associados a patógenos (PAMPs) que também podem ser detectados pelos astrócitos residentes(107). As consequências da liberação das citocinas podem variar de acordo com o tipo celular, incluindo a liberação de forma prolongada de mediadores inflamatórios, alteração da função da célula-alvo ou indução da morte celular (107). A maior ativação micróglial e/ou astrogliose com um aumento da liberação de citocinas pró inflamatórias (TNF-α, IL6 ou NF-KB) foram encontradas em modelos de hipertensão que podem estar envolvidas nos mecanismos de regulação da hipertensão (88,108,109).

O fenótipo de ativação da micróglia em resposta a hipertensão, danos ou inflamação tecidual permite que elas se adaptem, o qual é classicamente identificado pelo aumento da produção de citocinas, a adoção de uma morfologia ameboide e mudanças em marcadores proteicos relevante (110). No processo de ativação da micróglia, ela adapta seu formato ramificado para um formato mais espesso com um corpo celular mais arredondando conhecido como formato ameboide (111). Nesse fenótipo, a micróglia se torna mais ativa no processo de fagocitose de fragmentos de neurônios ou patógenos, secreta citocinas inflamatórias e quimiocinas e outras moléculas que realizam o recrutamento de outras células imunes para o local da lesão, amplificando a resposta inflamatória(112).

Os astrócitos desempenham funções cruciais tanto na neuroinflamação quanto na neuroproteção no sistema nervoso central (113). Eles secretam citocinas pró e anti-inflamatórias, além de substâncias que promovem ou inibem o crescimento neuronal. Essas células também ajudam a manter a homeostase sináptica, garantem a integridade da barreira

hematoencefálica e fornecem suporte metabólico aos neurônios. Além disso, os astrócitos protegem os neurônios contra o estresse oxidativo (114).

O mecanismo de astrogliose reativa ocorre em resposta a lesão no sistema nervoso central, na qual ocorre a movimentação dos astrócitos para o local da lesão, resultando tanto na proteção do tecido lesionado como do não lesionado (115). Dessa forma o processo de astrogliose reativa regulada é de suma importância para o cérebro, devido os seus efeitos neuroprotetores. A astrogliose reativa desregulada é um processo prejudicial que contribui para as doenças cardiovasculares (113). Dessa forma a ativação da micróglia e a astrogliose são componentes centrais da neuroinflamação contribuindo para hipertensão neurogênica, considerando que o aumento das citocinas pró-inflamatórias nos núcleos centrais de regulação da pressão arterial promove tanto o aumento do sistema nervoso simpático como da inflamação.

Dessa forma, o reconhecimento da hipertensão como um processo inflamatório sistêmico é fundamental, pois pode auxiliar tanto na compreensão da origem da doença quanto no desenvolvimento das complicações associadas a ela, considerando que sua gravidade contribui para o surgimento de disfunções cardiovasculares (70).

Justificativa

Este estudo possibilitou a identificação de mecanismos fisiopatológicos implicados na hipertensão, relacionada a insultos na vida precoce. O presente trabalho contribuiu para explorar a fase da peripuberdade, como uma fase crítica do desenvolvimento e esse conhecimento poderá fundamentar pesquisas focadas em intervenções precoces e tratamentos na peripuberdade que possam identificar fatores de risco bem como biomarcadores para a prevenção das doenças cardiovasculares. O conhecimento científico advindo dessa pesquisa poderá contribuir para a conscientização da população sobre a relevância da exposição à uma dieta balanceada nas fases sensíveis do desenvolvimento para prevenção de doenças cardiovasculares e importância de se fazer um acompanhamento cuidadoso da inflamação, função e estrutura cardíaca.

Objetivo geral

Avaliar a implicação da inflamação e do sistema renina angiotensina na hipertensão e alterações estruturais cardiovasculares a longo prazo induzida por restrição proteica na peripuberdade.

Objetivos específicos

- ✓ Avaliar os parâmetros murinométricos: ingesta alimentar, peso corporal, comprimento naso-anal e peso dos tecidos (coração, aorta e gorduras);
- ✓ Avaliar os parâmetros bioquímicos (glicemia, perfil lipídico e marcadores de lesão);
- ✓ Analisar a variabilidade da PA e do intervalo de pulso, no domínio da frequência (análise espectral) em animais acordados;
- ✓ Avaliar a sensibilidade espontânea do barorreflexo;
- ✓ Estudar a pressão arterial e frequência cardíaca em condições basais e em reposta a administração de drogas para avaliar o SRA;
- ✓ Avaliar os níveis circulantes de angiotensina 2 e angiotensina 1-7;
- ✓ Avaliar a expressão do mRNA no coração e aorta para os genes do SRA;
- ✓ Avaliar os parâmetros eletrocardiográficos em animais anestesiados;
- ✓ Avaliar os parâmetros ecocardiográficos em animais anestesiados;
- ✓ Analisar os parâmetros inflamatórios no coração e encéfalo.

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CAPÍTULO II

Artigo: Hypertension induced by peri-pubertal protein restriction depends on renin-angiotensin system dysfunction in adult male rats

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Research Paper

Hypertension induced by peri-pubertal protein restriction depends on renin-angiotensin system dysfunction in adult male rats

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ABSTRACT

Background and aims: Hypertension depends on renin-angiotensin system dysfunction; however, little is known about its implications in the outcomes of neurogenic hypertension induced by peri-pubertal insults. This study aimed to evaluate whether hypertension induced by a peri-pubertal low-protein diet is related to renin-angiotensin system dysfunction in adult male Wistar rats.

Methods and results: Thirty-day-old male Wistar rats were fed a low-protein diet (4 % casein) for 30 days and subsequently fed a 20.5 % normal protein diet for a 60-day dietary recovery (LP group). Control animals (NP group) were fed a 20.5 % protein diet throughout their lives. Cardiovascular and renin–angiotensin system functions were evaluated on postnatal day 120 (6–24 animals per group). Statistical analyses were performed using the Student's t-test. Animals with LP show increased arterial blood pressure. The angiotensin 2 dose-response curve of LP animals showed an increase in the pressor response at a lower dose (50 ng/kg) and a reduction in the pressor response at a higher dose (400 ng/kg) compared with NP animals. Angiotensin 2 type 1 receptor mRNA levels were increased in the hearts of LP animals; however, angiotensin 2 type 2 receptor and MAS receptor mRNA levels were reduced. In the aorta, AT1 and AT2 mRNA levels were increased in LP animals, whereas MAS receptor mRNA levels were decreased in comparison to NP animals.

Conclusion: The renin-angiotensin system is disrupted in hypertension induced by protein restriction exposure during peri-pubertal life.

1. Introduction

The World Health Organization estimates that 1.28 billion individuals worldwide are hypertensive, two-thirds of whom live in low- and middle-income countries [1]. Malnutrition is a risk factor for hypertension. A country's economic status is related to inequality in food access, which aggravates malnutrition and public health problems. By 2023, it is estimated that between 713 and 757 million people worldwide may have faced hunger, mostly in Asia, Africa, and Latin America [2].

Studies founded on the Developmental Origins of Health and Disease (DOHaD) concept provide evidence that exposure to environmental stresses, such as malnutrition, occurring during sensitive windows of development, increases susceptibility to noncommunicable chronic diseases in adulthood, including hypertension [3,4]. The peri-pubertal life phase is also a sensitive developmental window [5,6]. Previously, our group reported that adult Wistar male rats exposed to caloric protein restriction during peri-pubertal life have a metabolic syndrome characterized by increased visceral fat accumulation, hyperleptinemia, hyperphagia, dyslipidemia, glucose-insulin homeostasis disruption (characterized by hyperglycemia and hyperinsulinemia), hypertension,

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Abbreviations

LP	low protein
NP	animals fed a normal protein diet
Ang 2	angiotensin 2
AT1R	angiotensin 2 type 1 receptor
AT2R	angiotensin 2 type 2 receptor
MAS	MAS receptor
ACE1	angiotensin-converting enzyme 1
ACE2	angiotensin-converting enzyme 2
DOHaD	Developmental Origins of Health and Disease
RAS	renin-angiotensin system
PN	post-natal day
SAP	systolic arterial pressures
DAP	diastolic arterial pressures
MAP	mean arterial pressures
PI	pulse interval
HR	heart rate
SEM	standard error of the mean
SHR	spontaneously hypertensive rat
mRNA	messenger RNA
cDNA	complementary DNA
AU	arbitrary units

cardiac remodeling, cardiovascular autonomic dysfunction (characterized by sympathetic hyperactivity), and endothelial dysfunction [7–9].

Components of the circulating and local renin-angiotensin system (RAS) are involved in cardiovascular diseases, notably in the development and progression of hypertension and its comorbidities, such as cardiac remodeling [10,11]. Ang 2 is the main endogenous agonist of the Ang 2 type 1 receptor (AT1R), which mediates vasoconstriction, cell proliferation, oxidative stress, and hypertension [10,11]. Furthermore, evidence points to RAS dysfunction in hypertension following malnutrition in the post-weaning/peri-pubertal phase [12]. However, the long-term implications of the RAS in neurogenic hypertension induced by peri-pubertal caloric protein restriction have not been evaluated. This study hypothesized that adult hypertension induced by low-protein diet exposure in peri-pubertal life may be associated with RAS dysfunction.

2. Methods

The experimental protocol was approved by the Research Ethics Committee for Animal Use and Experimentation at the State University of Maringa (approval number 3353060421). Animals were housed in four rats per cage (polypropylene cages with 40 × 34 × 17 cm) under controlled temperature (22 ± 2 °C) and photoperiod (07:00–19:00, light cycle) conditions, with *ad libitum* access to water and food.

Twenty-five-day-old male Wistar rats obtained from the Central Animal Facility of the State University of Maringa were exposed to the same perinatal environment and maintained in the sectoral vivarium of the Department of Biotechnology, Genetics, and Cell Biology. As soon as they arrived at the sectorial facility, the animals were randomized considering their balanced body weight in all boxes and expended five days of adaptation before starting the dietary treatment at PN 30. During all the experimental protocols, only two experienced researchers manipulated the animals to minimize environmental stress.

At post-natal day (PN) 30, the animals were randomly assigned into two groups: those fed a palletized isocaloric low-protein diet (4 % protein; LP group; Table 1) or a balanced commercial control diet (20.5 % protein; Nuvital®, Curitiba/PR, Brazil; NP; Supplementary Table 1) until PN60. After the dietary manipulation period, all animals were fed the same commercial control diet (20.5 % protein; Nuvital®, Curitiba/PR, Brazil; NP; Table 1) for the remaining 60 days.

Table 1
Effects of dietary protein restriction in the peri-pubertal period on peptides circulating levels.

Biochemical parameters	NP	LP	P value
Endogenous Angiotensin 2 (pg/ml)	38 ± 1.91	52 ± 2.26	0.0001***
Endogenous Angiotensin 1-7 (pg/ml)	377 ± 4.07	375 ± 8.09	0.874

NP: Normal protein diet; LP: LP diet; n = 10 to 12 animals per group. Values expressed as mean ± SEM. **p < 0.01 and * p < 0.05, indicate statistical significance (Student's T test).

At PN120, both groups were evaluated for RAS function, biochemical (Supplementary Table 2) and biometric parameters (Supplementary Table 3), food intake (Supplementary Fig. 1), body weight (Supplementary Fig. 2), and mRNA expression of RAS components.

2.1. Biochemical parameters

Total blood was collected and serum was separated for biochemical analysis. Glycemia was quantified by the glucose oxidase method by spectrophotometry in microplates (SpectraMax® Plus 384 Microplate Reader from Molecular), using a commercial kit (Gold Analisa®, Belo Horizonte/MG, Brazil). The details are provided in the Supplementary Methodology. The results were expressed in mg/dL.

Plasma quantification of endogenous circulating Ang 2 and Ang 1–7 was performed using a rat ELISA kit (Elabscience/Wuhan Fine Biotech Co., Ltd/Catalogue No.: E-EL-R1430 and FineTest/Wuhan Fine Biotech Co., Ltd/Catalogue No.: ER1616).

2.2. Arterial and venous catheterization

After anesthesia (ketamine-xylazine; 75 mg + 15 mg/kg, i.p.), two cannulae (P10 -Micro-Renathane connected to P50 cannulae (ClearTygon)) were inserted, one into the femoral artery for monitoring arterial pressure, and one in the femoral vein for drug administration. At the end of the surgery, the animals received anti-inflammatory analgesics (Meloxican, 0.4 mg/kg, i.v.) and antibiotics (enrofloxacin 5 mg/kg, i.v.). The lines were heparinized (250 U/mL) after surgery, and the animals were kept in individual boxes for recovery. After 48 h of recovery [13–16], the assessment of RAS modulation was performed over three days.

2.3. Arterial pressure recording

After 1 h of room adaptation, baseline arterial pressure was recorded for 30 min on each of the three days of the experimental protocol. Following baseline recordings, intravenous injections were administered according to the protocol described below. Experiments were performed on conscious, free-moving animals between 10 a.m. and 3 p.m. Recordings were sampled at 1000 Hz. The details are provided in the Supplementary Methodology.

Data were pre-analyzed using Advanced CODAS software (Data Q instruments, United States, https://www.dataq.com/products/windaq/, paid software) to objectively identify stable periods of recording and to exclude erratic signals. Baseline systolic (SAP), diastolic (DAP), mean arterial pressure (MAP), and pulse interval (PI) values were transferred to a spreadsheet for analysis (Microsoft Excel, Redmond, WA, EUA) over 10 min of recording under stable conditions. The heart rate (HR) was calculated from the pulse interval (ms) using formula (60000/PI in ms).

2.4. Effect of angiotensin-converting enzyme inhibitor

On the first day of the blood pressure evaluation, baseline recordings of the arterial pressure were performed over 30 min. Enalapril (5 mg/kg; intravenous; Sigma-Aldrich, San Luis, MO, EUA), an angiotensin-converting enzyme (ACE) inhibitor, was used to estimate the effect of ACE on arterial pressure [12,17–19]. The arterial pressure and heart rate were recorded for 30 min after injection, and the response was calculated from the MAP and HR baseline.

2.5. Effect of angiotensin 2

On the second and third days of the protocol, following baseline recordings, a range of doses of Ang 2 (50, 200, and 400 ng/kg; i.v.; Sigma-Aldrich, San Luis, MO, EUA) was injected into different NP and LP animals according to the rat body weight [12,20]. To avoid receptor desensitization, only one dose of Ang 2 was performed to each animal on each experimental day and only two doses were injected in each animal, with 24 h of interval. MAP and HR responses were calculated from the baseline.

2.6. mRNA expression of RAS components in the heart and thoracic aorta

In a separate cohort of animals, samples of the left ventricle and thoracic aorta were collected after euthanasia using guillotine. Tissues were frozen in nitrogen and subsequently stored at −80 °C for molecular analyses. Total RNA was extracted using TRIzol™ (Invitrogen®) following the manufacturer's instructions. Total RNA concentration was measured spectrophotometrically at 260 nm (NanoDrop ND 1000 NanoDrop Technologies; Wilmington, DE, USA). For cDNA synthesis 2 μg of RNA was converted into cDNA using a high-capacity cDNA reverse transcription kit (Promega US, Madison, WI, USA), and samples were run using an AcessQuick™ Master Mix (Promega US, Madison, WI, USA). RT-PCR was performed using the Promega Access RT-PCR System (Promega US, Madison, WI, USA). The first strand of cDNA synthesis was obtained by one cycle of 5 min at 25°C, followed by one cycle of 1 min at 42 °C and one cycle of 15 min at 70 °C. The sample was then sequentially thermocycler at 95°C for 2 min, followed by 40 cycles of 15 s at 95 °C and 40 cycles of 1 min at 60 °C. The expression patterns of genes of interest were normalized to constitutively expressed beta-actin and the primers used were described (Supplementary Table 4). The $2^{-\Delta CT}$ method was used for the relative quantification analysis and data were expressed in arbitrary units (AU) [21,22].

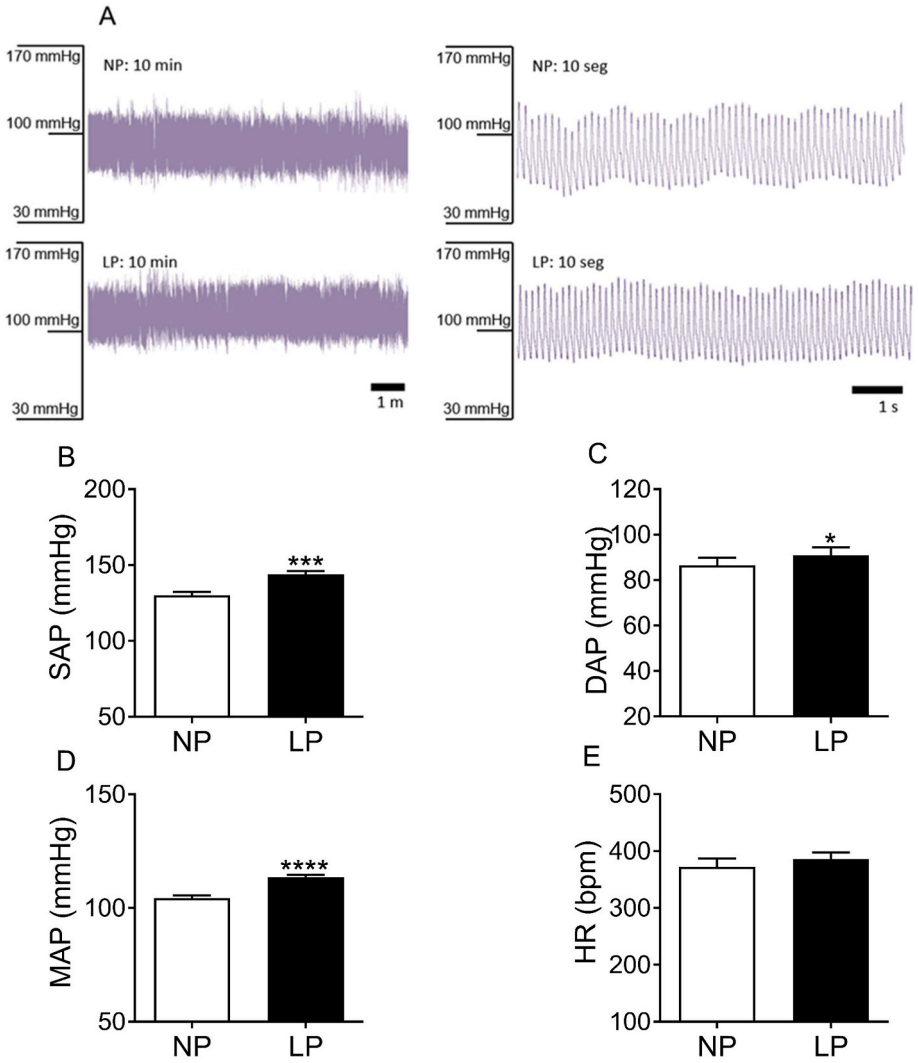

Fig. 1. Basal arterial pressure. Representative arterial pressure traces of NP and LP animals in basal conditions (A), systolic arterial pressure (B), diastolic arterial pressure (C), mean arterial pressure (D), and heart rate (E). n = 9–13 animals per group. NP, normal protein diet; LP, low protein diet. Values presented as mean ± SEM. ***p < 0.001 and * p < 0.05, indicate statistical significance (Student's T test).

2.7. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed after analysis of data distribution (Shapiro-Wilk test). Results were considered significant at $P < 0.05$. GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA (https://www.graphpad.com/scientific-software/prism/); was used for graphical representations and statistical analyses. Comparison between the groups was performed using Student's T-test, Mann-Whitney test, or two-way ANOVA with time, dose, and diet as independent factors.

3. Results

3.1. Biochemical parameters

The endogenous circulating Ang 2 was increased in LP animals (LP: 52 ± 2.26 vs. NP: 38 ± 1.91 pg/ml, p = 0.0001) compared with NP animals, but endogenous Ang 1–7 was similar between groups (Table 1).

3.2. Arterial pressure, pulse pressure, and heart rate

Representative arterial blood pressure traces are shown in Fig. 1A. LP animals showed increased SAP (LP: 144 ± 1.91 vs. NP: 129 ± 2.21 mmHg, p = 0.0002; Fig. 1B), DAP (LP: 90 ± 1.04 vs. NP: 86 ± 1.11 mmHg, p = 0.010; Fig. 1C) and MAP (LP: 113 ± 0.97 vs. NP: 104 ± 1.10 mmHg, p = 0.001; Fig. 1D) compared with NP animals. A similar HR was observed between groups (Fig. 1E).

3.3. Effects of enalapril on MAP and HR

LP animals showed a reduced MAP response to the ACE inhibitor, enalapril (LP: 7.25 ± 1.14 vs. NP: 12.49 ± 1.21 mmHg, p = 0.007; Fig. 2A) compared with the NP group. The HR response was similar between groups (Fig. 2B).

3.4. Effects of angiotensin 2 in MAP and HR

Fig. 3 shows the dose-response curves of MAP and HR for Ang 2. In the lower dose (50 ng/kg), the LP animals had a greater MAP response (LP: 57 ± 6.28 vs. NP: 30 ± 5.39 mmHg, p = 0.009; Fig. 3A) compared with the NP group; while the delta of HR was reduced in LP compared with NP animals (LP: 42 ± 27.68 vs. NP: 35 ± 18.97 bpm; p = 0.049, Fig. 3B). In the two highest doses of Ang 2 (200 and 400 ng) the LP animals showed attenuated increase of the MAP (LP: 41 ± 3.67 vs. NP: 64 ± 4.47 mmHg, p = 0.039, and LP: 49 ± 6.66 vs. NP: 74 ± 4.16 mmHg, p = 0.011, respectively; Fig. 3A) compared with the NP group; while the HR response in these doses was similar between the groups (Fig. 3B).

3.5. mRNA expression of RAS components in the heart and thoracic aorta

Fig. 4 shows the mRNA expression of the RAS markers in the heart and thoracic aorta. LP animals had increased levels of angiotensin-converting enzyme 1 (ACE1) and AT1 mRNA in the heart (LP: 3.40 ± 0.99 vs. NP: 0.85 ± 0.25 AU, p = 0.020; and LP: 4.66 ± 1.70 vs. NP: 0.97 ± 0.21 AU, p = 0.019, respectively; Fig. 4A) compared with NP group. The mRNA levels of angiotensin-converting enzyme 2 (ACE2), AT2, and MAS in the heart were lower in LP compared with NP animals (LP: 0.70 ± 0.17 vs. NP: 1.36 ± 0.23 AU, p = 0.040; LP: 0.68 ± 0.17 vs. NP: 2.42 ± 0.78 AU, p = 0.029; and LP: 1.11 ± 0.29 vs. NP: 2.58 ± 0.53 AU, p = 0.027, respectively; Fig. 4A).

In the aorta, the ACE1 and AT1 mRNA levels were increased in LP compared with NP animals (LP: 1.51 ± 0.43 vs. NP: 0.43 ± 0.12 AU, p = 0.005; and LP: 2.69 ± 0.56 vs. NP: 1.14 ± 0.36 AU p = 0.036, respectively; Fig. 4B). In the contra regulatory via, the LP animals showed a decrease in mRNA levels of ACE2 and MAS respectively (LP: 0.76 ± 0.29 vs. NP: 2.38 ± 0.68 AU, p = 0.045; and LP: 0.48 ± 0.14 vs. NP: 1.34 ± 0.33 AU, p = 0.013, respectively; Fig. 4B) but mRNA levels of AT2 in LP animals were increased (LP: 4.23 ± 1.17 vs. NP: 1.71 ± 0.33 AU, p = 0.023; Fig. 4B) compared with the NP group.

4. Discussion

This work demonstrates, for the first time, that caloric protein restriction in the peri-pubertal period followed by 60 days of nutritional recovery induces RAS dysfunction in adulthood. The present findings appear to be underpinned by the hyperactivation of Ang 2 and attenuation of its contra-regulatory via. Other studies in the DOHaD context have pointed to RAS dysfunction in adult hypertensive rats programmed by the LP diet in perinatal life [17,23,24]. The main finding of the present study is that hypertension, observed in LP adult animals programmed during peri-pubertal life, depends on RAS hyperfunction, as evidenced by: 1) increased endogenous circulating Ang 2; 2) overexpression of ACE1 and AT1 mRNA and decreased expression of ACE2 and MAS mRNA in the heart and aorta; and 3) increased pressor response to the lower dose of Ang 2 (50 ng/kg).

During basal conditions, LP animals showed increased blood pressure and greater levels of endogenous circulating Ang 2, produced by the cleavage of angiotensinogen by the ACE1. The present animals showed increased expression of ACE1 mRNA, which may suggest that ACE1 protein is overexpressed in LP animals, driving the increased levels of Ang 2. It is well known that the main effects of Ang 2 on blood pressure are mediated by the AT1R, including, among other mechanisms, vasoconstriction and water retention in the kidney, leading to hypertension [11,25]. The LP animals also showed increased expression of AT1 mRNA, suggesting that these animals express more AT1R. However, it is important to consider that mRNA is not a determinant of protein

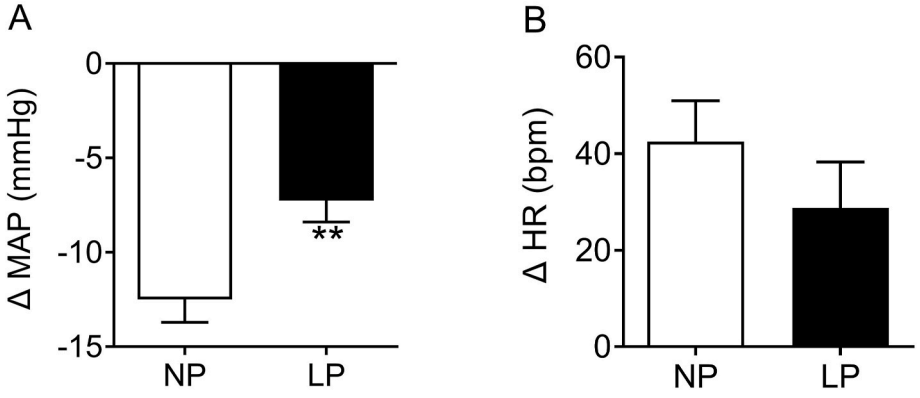

Fig. 2. Pressure response to enalapril. Change in mean arterial pressure (A) and change in heart rate in response to enalapril (B). n = 7 to 8 animals per group. NP, normal protein diet; LP, low protein diet. Values presented as mean ± SEM. **p < 0.01 indicate statistical significance (Student's T test).

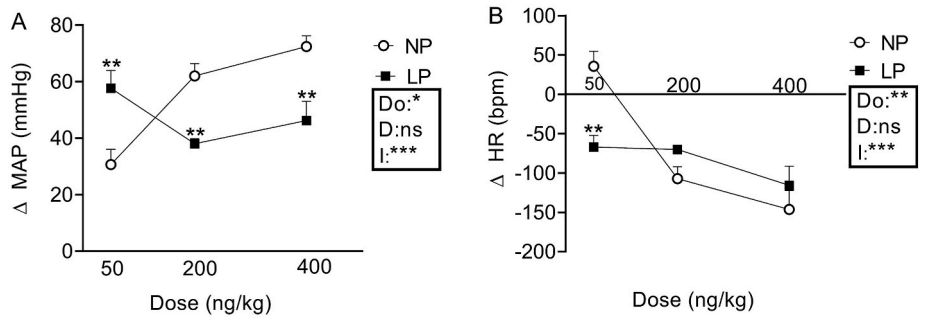

Fig. 3. Blood pressure response to angiotensin 2. Change in mean arterial pressure (A) and change in heart rate in response to angiotensin 2 (B). n = 5 to 9 animals per group. NP, normal protein diet; LP, low protein diet; I, interaction between diet and dose. Values presented as mean ± SEM. **p < 0.01 and * p < 0.05, indicate statistical significance (Two-way ANOVA).

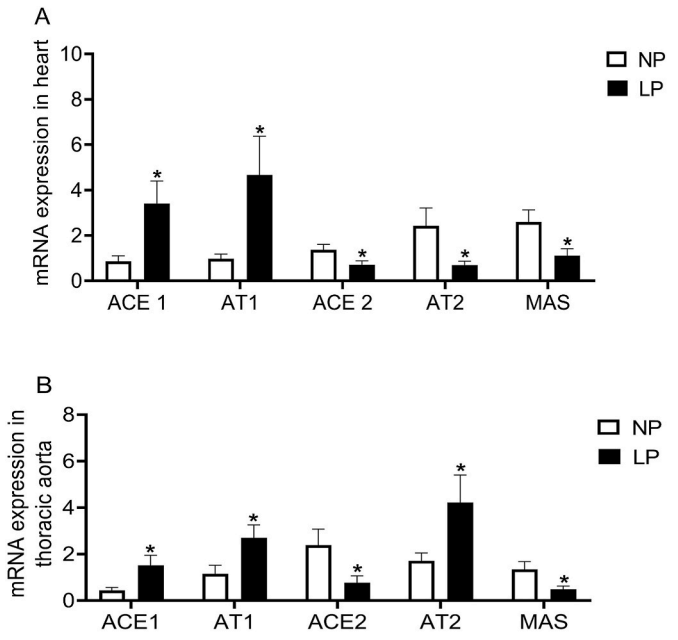

Fig. 4. RAS mRNA expression in the heart and thoracic aorta. mRNA expression of RAS components in the heart (A), mRNA expression of RAS components in the thoracic aorta (B), n = 6 to 10 animals per group. NP, normal protein diet; LP, low protein diet; ACE 1: Angiotensin-converting enzyme 1; ACE 2: Angiotensin-converting enzyme 2; AT1: Angiotensin 2 type 1 receptor; AT2: Angiotensin 2 type 2 receptor; MAS: MAS receptor. Values presented as mean ± SEM. *p < 0.05, indicate statistical significance (Student's T test or Mann-Whitney test).

expression, and the extrapolation of the data from mRNA to protein expression needs to be considered with caution. Unfortunately, AT1R expression was not explored in the present study due to methodological issues[26–30]. It has been pointed out that commercial antibodies also target other receptors coupled to the G protein and cross-react with muscarinic, adrenergic, and dopamine receptors[31–35]. The production of antibodies with high specificity for G protein-coupled receptors is difficult; therefore, we used one of the methods described in the literature to study the biology of AT1R [29]. AT1R activation also induces dysregulation of oxidative stress [23,36] and cardiac hypertrophy [37]. We have previously shown that the present model of peri-pubertal LP diet exposure induced cardiac hypertrophy, associated with interstitial fibrosis and disorganization of the cardiac redox state in adulthood [7]. Collectively, these findings indicate that neurogenic hypertension may be related to unbalanced AT1R function and its contra regulatory via.

When LP animals were challenged by increasing doses of Ang 2, a greater blood pressure response was observed to a low dose of Ang 2 (50 ng/kg/iv), which was attenuated at higher doses (200 ng e 400 ng/kg/iv). This pattern of blood pressure response to exogenous Ang 2 corroborates previous data from the literature. Gomide et al. (2013) showed that exposure to the LP diet after weaning (PN28 until PN63) induced an increased pressor response to a lower dose of exogenous Ang 2 (doses: 100 ng/kg, i.v.) and an attenuation in the blood pressure response to the higher doses of exogenous Ang 2 (doses: 200, 300, and 400 ng/kg, i.v.), just after the end of the special diet exposure, at PN64 [12]. Although the pattern of response was similar in the two studies, our study evaluated blood pressure after 60 days of dietary recovery. We may suggest that this outcome may be a consequence of AT1R desensitization. It has been shown a rapid desensitization and internalization of AT1R upon agonist stimulation, which is more evident in hypertension [38,39]. Several mechanisms mediate the desensitization to the Ang 2 signal, such as the recycling of the receptor, Ang 2-induced internalization of receptors [40], PKC-induced serine phosphorylation of AT1R and AT2 receptor (AT2R) [41] and the induction of the early inducible genes, c-fos and c-jun [42]. Furthermore, in spontaneously hypertensive rats (SHR) or hypertensive rats programmed in perinatal life [43,44], the intracellular machinery for AT1R function may be disrupted, which may be involved in AT1 desensitization [43,44]. Mesquita and collaborators (2010) showed that the intracellular signaling of the AT1R is disrupted in the kidneys of adult rats exposed to a perinatal LP diet [44]. This is a pathway that also needs to be explored in the present model.

The LP animals showed an attenuated blood pressure decrease in response to enalapril; however, the mechanism mediating this pattern is unclear. It appears that ACE1 is overexpressed/overactivated in the LP animals, as they show elevated levels of circulating endogenous Ang 2 and increased expression of ACE1 mRNA. We may suggest that the dose of enalapril used to inhibit ACE1 was insufficient to induce a similar ACE1 inhibition in the LP and control animals. An attenuated depressor response to enalapril was also observed in an animal model of streptozotocin-induced diabetes, which also showed increased endogenous circulating ACE1 [45]. Some studies suggest that the attenuated depressor response to the Enalapril observed in the animal model of streptozotocin-induced diabetes, may depend on the elevated concentration of circulating ACE, thus the same dose of enalapril may be less effective in inhibiting the transformation of Ang 1 to Ang 2 [46,47]. In both models, it may be suggested that a greater expression of ACE1 may be driving the observed increased production of Ang 2 and its effect; however, more studies using higher doses of enalapril are needed to better explore the implications of ACE1 in the present hypertension.

Although the depressor response to enalapril was attenuated in the LP animals, the HR was not significantly different between the groups. The absence of a difference may or may not be dependent on the variance between animals, as the variance in HR was greater than that in BP. It is important to consider that tachycardiac and depressor responses to enalapril were 33 % and 42 % lower, respectively, in LP animals and control animals. The present data do not support an attenuated

tachycardic response to enalapril in LP animals; however, LP animals could have an attenuated tachycardiac reflex because of attenuated hypotension, and more studies need to be performed to better explore this issue. Other peptides regulated by ACE1 that affect the heart rate and sympathetic nervous system independent of Ang 2, such as bradykinin, could be enrolled in the present findings [48]. Therefore, further studies are necessary to better understand the tachycardic response to enalapril.

The mRNA levels of ACE2, AT2, and MAS, which are collectively termed as "protective arm" of the RAS, were reduced in the heart of the present neurogenic hypertensive animals. It is well known that AT2R and MAS activation promote vasodilatation and diuresis/natriuresis, counterbalancing AT1R-mediated effects [49]. SHR is a genetic model of neurogenic hypertension with reduced cardiac ACE2 and MAS expression in the heart [50]. Another model of programmed hypertension, induced by perinatal exposure to an LP diet, also showed reduced AT2R expression in the left ventricle [51]. It has been suggested that the expression of AT2R can be tissue-dependent and/or dependent on the pathophysiological state of hypertension, showing increased, decreased, or unchanged expression[52–54]. In our study, we observed that the LP animals had increased AT2 mRNA levels in the aorta. The SHR also shows increased aortic AT2 mRNA levels, which appears to be associated with hypertrophy of the smooth muscle cells [52,54]. Furthermore, the aortic overexpression of the AT2R acts as a compensatory mechanism to vasoconstrictor agents, such as Ang 2, or overexpression of the AT1R. Additional studies need to be performed to better explore the role of the AT2R in this model.

Hypertension may be driven by several simultaneous mechanisms [55]. Previously, we have shown that peripubertal exposure to low protein diet programs long-term hypersympathetic activity, which may drive hypertension in adulthood [7]. In the present study, we showed that the disruption of RAS is also enrolled in the physiopathology of the present model of programmed hypertension [7]. It is well known that Angiotensin 2 can increase blood pressure via vasoconstriction, baroreflex dysfunction, and sympathetic activation [56]. Studies from the 60s already showed that the increase in circulating angiotensin 2 promotes an increase in the sympathetic nervous system [57]. Furthermore, the renal sympathetic activity facilitates the renin release in the juxtaglomerular apparatus [58,59]. The RAS system and sympathetic nervous system, booths can activate common pro-hypertensive mechanisms, as the increase in vascular resistance and endothelial dysfunction [59,60]. The connecting link between the RAS and the sympathetic nervous system was not explored in the present study, however, it is important to examine this issue in future studies.

The present findings point to the dysregulation of the RAS in adult rats, which express neurogenic hypertension induced by peri-pubertal exposure to caloric protein restriction. Elevated endogenous circulating Ang 2 levels may mediate the hypersympathetic activity observed in animals with LP [7]. However, RAS has efficient mechanisms of counterbalancing Ang 2 effects, such as AT1R desensitization, which may have occurred in the present LP rats and attenuated the pressure response to higher doses of Ang 2. Furthermore, the "protective arm" of the RAS was attenuated in the LP animals, which may be contributing to the increased blood pressure levels observed in these animals. In summary, this conclusion is underpinned by the greater effects of Ang 2 and a decrease in contra-regulatory activity, as indicated by the reduced mRNA expression of ACE2, AT2, and MAS. This study provides a better understanding of the mechanisms involved in peri-pubertal insult-induced neurogenic hypertension. Additionally, these findings point to potential pharmacological interventions, such as the regulation of RAS function, to control the increase in blood pressure in hypertensive adults exposed to developmental insults such as peri-pubertal caloric protein restriction. Furthermore, it is important to consider the relevance of adequate public health interventions in the peri-pubertal life period to prevent the long-term development of chronic cardiometabolic dysfunction, as observed in the present animal model.

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Author contributions

Anna Ferreira and Kesia Palma-Rigo: Contributed to the conceptualization of the study project, data curation, formal analysis, and writing of the manuscript. Anna Ferreira, Maiara Ribeiro, Maria Peres, Gabriel Lopes, Silvano Piovan, Lucas Saavedra, Leticia Barbosa, Scarllet Raposo, Ananda Malta, and Douglas Almeida: Contributed to the performance of the investigation, methodology, and data curation. Paulo Mathias and Jorge Teixeira: Contributed to reviewing and interpreting the results and funding acquisition.

Declaration of competing interest

The authors declare no potential conflict of interest.

Acknowledgments

Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.numecd.2024.09.003.

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Supplementary materials

Supplementary Table 1 - Composition of the isocaloric low-protein and normal-protein diets.

Diet components	Normal protein (20.5 %)	Low protein (4.0 %)
Sucrose	12.72	20.00
Corn starch	52.75	64.25
Casein (88% protein)	23.33	4.55
Mix of mineral salts	3.20	3.20
Mix of vitamins	1.60	1.60
Soybean oil	4.80	4.80
Fish oil	1.60	1.60
Total (g)	100.0	100.0

Supplementary Table 2 – Effects of dietary protein restriction in peri-pubertal period on biochemical parameters.

Biochemical parameters	NP	LP	P value
Blood glucose (mg/dL)	105 ± 4.28	115 ± 1.99	0.045*
Triglycerides (mg/dL)	97 ± 8.01	127 ± 10.11	0.044*
Total cholesterol (mg/dL)	92 ± 5.54	88 ± 4.34	0.560
HDL - cholesterol (mg/dL)	37 ± 3.25	39 ± 3.81	0.728
Triglyceride-Glucose Index (TyG)	5.09 ± 0.47	7.52 ± 0.55	0.004**

NP: Normal protein diet; LP: Low protein diet; n = 10 to 12 animals per group. Values expressed as mean ± SEM. ** $p < 0.01$ and * $p < 0.05$, indicate statistical significance (Student's T test).

Supplementary Table 3 – Effects of dietary protein restriction in peri-pubertal period on biometric parameters

Biometric parameters	NP	LP	P value
Body weight (g)	411 ± 6.45	372 ± 2.73	0.0001***
Naso-anal length (cm)	23.64 ± 0.20	22.71 ± 0.20	0.010*
Retroperitoneal fat pad (g/100g bw)	1.28 ± 0.08	1.52 ± 0.05	0.028*

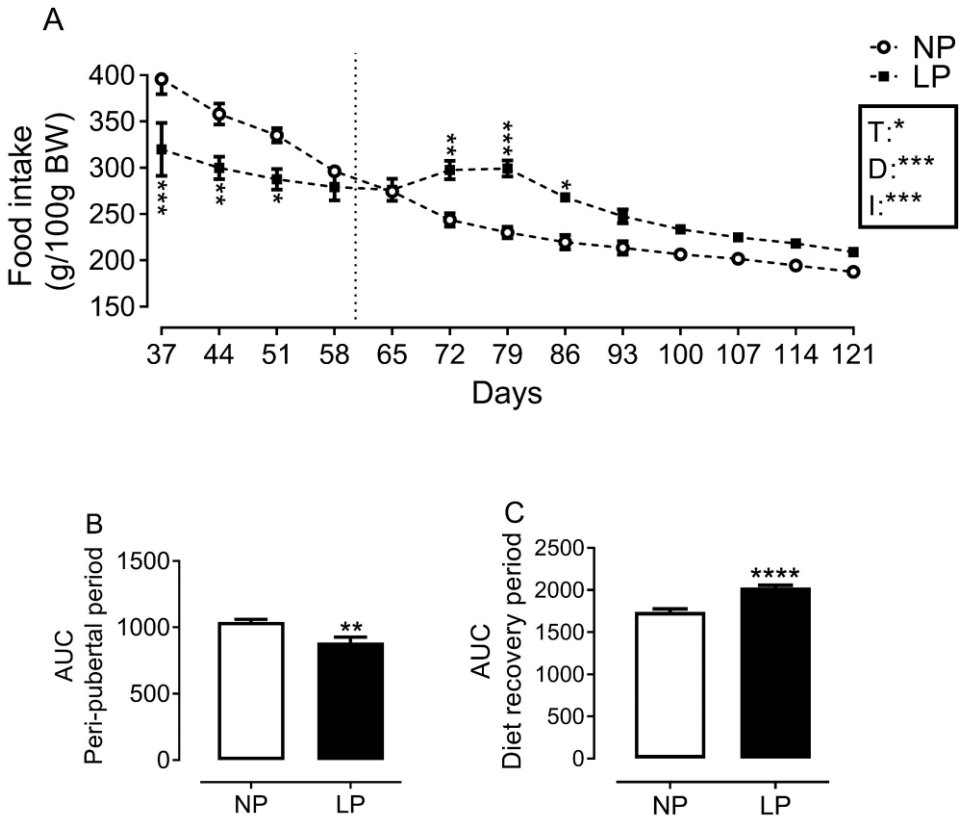
Mesenteric fat pad (g/100g bw)	0.77 ± 0.04	0.80 ± 0.02	0.403
Heart (g/100g bw)	0.51 ± 0.010	0.54 ± 0.006	0.001**
Thoracic aorta (g/100g bw)	0.050 ± 0.002	0.055 ± 0.0009	0.012*

NP: Normal protein diet; LP: Low protein diet; n = 9 to 17 animals per group.

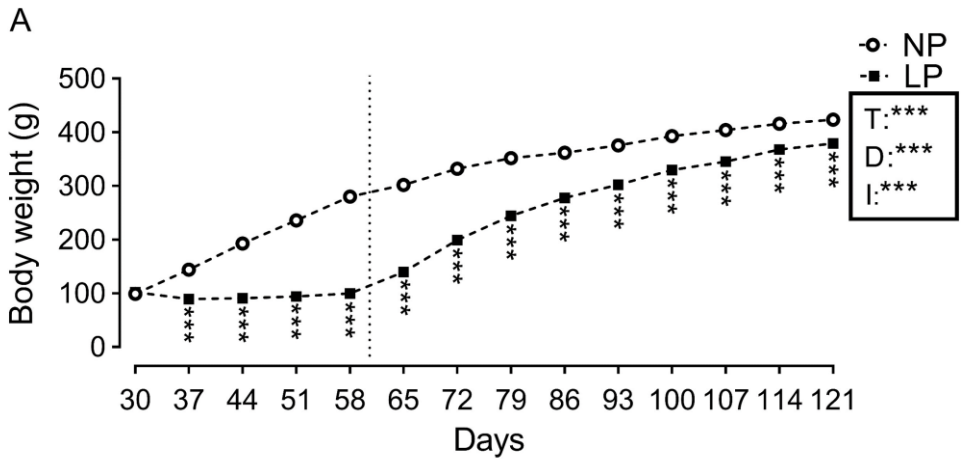
Supplementary Table 4 – List of primers used for RT-PCR.

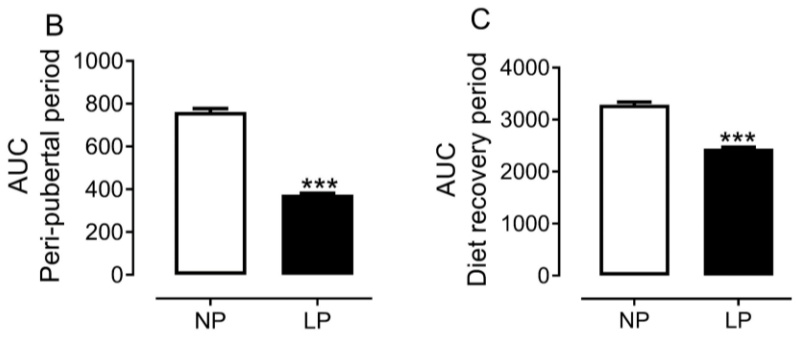
Primers	Sequence (5'-3') Forward	Sequence (5'-3') Reverse
Beta actin	5' GTCGTACCACTGGCATTGTG 3'	5' CTCTCAGCTGTGGTGGTGAA 3'
ACE1	5' GCTTGACCCTGGATTGCAGC 3'	5' CTCCGTGATGTTGGTGTCGT 3'
ACE2	5' GCCCAAAAGATGAACGAGGC 3'	5' GACGCTTGATGGTCGCATTC 3'
AT1	5' CTCTGCCACATTCCCTGAGTTA 3'	5'CACTTTCTGGGAGGGTTGTGT 3'
AT2	5' GGTCTGCTGGGATTGCCTTA 3'	5' GGAAGGGAAGCCAGCAAATG 3'
MAS	5′ CAATCGTGACGTTATCGGTG 3′	5′ TCTCTCCACACTGAT GGCTG 3′

ACE 1: Angiotensin-converting enzyme 1; ACE 2: Angiotensin-converting enzyme 2; AT1: angiotensin 2 type 1 receptor; AT2: angiotensin 2 type 2 receptor; MAS: MAS receptor.



Supplementary Figure 1 - Food intake. Food consumption (A), AUC food consumption during the peri-pubertal period (B), AUC food intake during the dietary recovery period (C), n = 20 to 24 animals per group. NP, normal protein diet; LP, low protein diet; I, interaction between diet (D) and time (T).





Supplementary Figure 2 - Body weight. Body weight through the experimental protocol. (A), AUC of body weight during the peri-pubertal period (B), AUC of body weight during the dietary recovery period (C). n = 20 to 24 animals per group. NP, normal protein diet; LP, low protein diet; I, interaction between diet (D) and time (T).

Supplementary methodology

2.1 Body composition and food intake

Food consumption was assessed three times per week. Weekly food intake was calculated according to the following formula: [absolute consumption of the week x 100/average weekly weight of the box] (Supplementary Fig. 1). Body weights were recorded weekly (Supplementary Fig. 2).

At PN120, a cohort of animals were fasted overnight and euthanized using guillotine. The body weight and nasoanal length were also measured. Tissues (fat deposits, heart, and thoracic aorta) (Supplementary Table 2) and blood samples were collected).

2.2 Biochemical parameters

Total cholesterol was measured using the colorimetric method of cholesterol oxidase and triglycerides using the colorimetric method of glycerol-3-phosphate oxidase using commercial kits (Gold Analisa®, Belo Horizonte / MG, Brazil). Both readings were performed in spectrophotometry equipment for microplate (SpectraMax® Plus 384 Microplate Reader by Molecular).

HDL-cholesterol was determined after the precipitation of chylomicrons and low-density lipoproteins with a commercial kit (Gold Analisa®, Belo Horizonte / MG, Brazil) and subsequent determination of HDL-cholesterol using the method described above for the measurement of total cholesterol.

2.3 Arterial pressure recording

The arterial cannula was connected to a pressure transducer (MLT0670, ADInstruments, Dunedin, New Zealand) connected to a recording system (Insight, Ribeirão Preto, Brazil) that used the DI 158 System for signal acquisition and analog-to-digital signal conversion (WinDaq lite, DataQ instruments, United States of America).

To minimize manipulation stress, the animal boxes were not changed on the day of blood pressure recordings, and the animals expended one hour in an isolated recording room for adaptation before blood pressure recordings. The animals were kept free-moving and exposed to minimal handle manipulation, and external noise and odors were avoided during the blood pressure measurement protocol.

Artigo: Hypertension induced by peri-pubertal malnutrition is mediated by meta-inflammation in male adult rats

Hypertension induced by peripubertal malnutrition is mediated by meta-inflammation in male adult rats

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Abstract

Background. Hypertension is related to meta-inflammation and cardiac dysfunction. Furthermore, peripubertal caloric-protein restriction induces neurogenic hypertension and cardiac dysfunction in adulthood; however, little is known about the physiopathology of this health condition.

Objective. Evaluate whether metabolic inflammation is enrolled in hypertension and cardiac dysfunction in adult rats, induced by a peri-pubertal low-protein diet exposure.

Methods. Male *Wistar* rats were initially fed a low-protein diet (4% casein) from post-natal day (PND) 30 to 60, followed by 60 days of recovery with a normal protein diet (20.5%) (LP group). Control animals (NP group) were continuously fed the 20.5% protein diet throughout their life. Cardiovascular function and inflammatory biomarkers were evaluated on PND 120 (5–28 /group). Statistical analyses were performed using the Student's t-test.

Results. Blood pressure was increased in the LP animals with an increase in heart damage markers, creatine kinase, and lactate dehydrogenase. In the heart, the LP group showed an increase in the thickness of the interventricular septum, internal diameters, thickness of the left ventricle, and the shortening fraction. In the electrocardiogram, LP rats also showed increased cardiac sympathetic activity, TNF-α, and IL6 mRNA were also increased in the heart. In the brain, LP animals showed an increased number of Iba-1 positive cells in the paraventricular nucleus of the hypothalamus, the intermediate nucleus of the solitary tract (iNTS), and the rostral ventrolateral medulla, along with increased GFAP immunoreactivity in the commissural NTS and iNTS.

Conclusions. The meta-inflammation may mediate adult neurogenic hypertension and cardiac dysfunction induced by peripubertal protein restriction.

Keywords: inflammation, protein restriction, heart, neuroinflammation, cardiometabolic programming.

1 Introduction

According to the first WHO Global Report (1), hypertension and its associated complications, affect more than 1 billion people worldwide, and become an urgent public health concern, contributing to premature death (1,2). For decades, cardiovascular disease has been the leading cause of death in the world (3). In 2022, recent dates from the Center for Disease Control (CDC) database show more than 7 thousand people died from a cardiovascular condition (4). Malnutrition is a risk factor for cardiovascular disease. It is estimated that, in 2023, between 713 and 757 million people worldwide may have faced hunger, mostly in Africa, Latin America, and the Caribbean (5).

Studies in the context of the Developmental Origins of Health and Disease (DOHaD) concept provide evidence that exposure to early life insults, such as malnutrition, increases susceptibility to noncommunicable chronic diseases in adulthood, including hypertension and other associated cardiovascular and metabolic diseases (6–10). The peri-pubertal life phase is one of the sensitive phases of development (6,7,11–14). Previously, our group reported that rats exposed to peri-pubertal caloric-protein restriction showed cardiometabolic syndrome, characterized by increased visceral fat accumulation, hyperleptinemia, hyperphagia, dyslipidemia, hyperglycemia, hyperinsulinemia, and hypertension, as well as reproductive dysfunction in adult life (6,7,10,15). Present cardiovascular dysfunction also appears to implicate endothelial dysfunction and cardiac remodeling, which may be mediated by cardiovascular sympathetic hyperactivity and renin-angiotensin system dysfunction (6,7). In addition to these results, we also observed oxidative stress associated with some of these dysfunctions (6,7,13) , which may be related to a systemic sterile chronic low-grade inflammation, or the meta-inflammation(16).

Meta-inflammation refers to persistent inflammation in the absence of infection, sustained by bilateral crosstalk between immune regulation and aberrant metabolism (17). The hypertension and cardiac remodeling present in the metabolic syndrome appear to be mediated by meta-inflammation (18). Studies have shown the implication of neuroinflammation in neurogenic hypertension and cardiovascular dysfunctions (19–21). Also, the increase in circulating pro-inflammatory cytokines and heart and brain inflammation is associated with a blood pressure increase (19,22,23). Furthermore, the chronic low-grade inflammation observed in non-communicable diseases may be mediated by early-life insults (24). This may point to a meta-inflammation composing the physio-pathological mechanism of hypertension associated with metabolic syndrome induced by early-life insults (25). However, little is known about the implication of meta-inflammation and neuroinflammation in hypertension programmed by peri-pubertal malnutrition. We hypothesize that the cardiovascular dysfunction and neurogenic hypertension induced by peri-pubertal caloric-protein restriction implicates meta-inflammation, characterized by neuro and cardiac inflammation.

2 Methodology

The study was approved by the Research Ethics Committee for Animal Use and Experimentation at the State University of Maringa (number 3353060421). Male Wistar rats obtained from the Central Animal Vivarium of the State University of Maringa were kept in the Sectorial Vivarium of the Department of Biotechnology, Genetics, and Cell Biology for five days of adaptation. The rats were housed in four per cage (polypropylene cages with 40 x 34 x

17 cm) under controlled temperature (22± 2 ºC) and photoperiod (07:00 - 19:00, light cycle) conditions, with *ad libitum* access to food and water.

At post-natal day (PN) 30, the animals were randomly assigned into two groups: those fed a palletized isocaloric low-protein diet (4% protein; LP group; Supplementary Table 1) or a balanced commercial control diet (20.5% protein; Nuvital®, Curitiba / PR, Brazil; NP; Supplementary Table 1) until PN60. After the dietary manipulation period, all animals were fed the same commercial control diet (20.5% protein; Nuvital®, Curitiba / PR, Brazil; NP; Table 1) for the remaining 60 days.

At PN120 both groups underwent evaluation of the biochemical and biometrical parameters, stress response, cardiovascular function, and inflammation in the heart and brain.

2.1 Body composition and food intake

Food consumption was assessed three times a week. Weekly food intake was calculated according to the formula: [absolute consumption of the week x 100/average weekly weight of the box] (Supplementary Fig. 1). Bodyweight was recorded every week (Supplementary Fig. 2).

At PN120, a cohort of animals was fasted overnight and then euthanized with a guillotine. Bodyweight and naso-anal length were evaluated. Tissues (fat depots and heart) (Supplementary Table 2) and blood samples were collected.

2.2 Biochemical and electrolyte analyses

Total blood was collected, and the serum was used for biochemical dosages. Glycemia was quantified by the glucose oxidase method by spectrophotometry in microplates (SpectraMax® Plus 384 Microplate Reader from Molecular), using a commercial kit (Gold Analisa®, Belo Horizonte / MG, Brazil). Total cholesterol was measured by the colorimetric method of cholesterol oxidase and triglycerides were measured using the colorimetric method of glycerol-3-phosphate oxidase using commercial kits (Gold Analisa®, Belo Horizonte / MG, Brazil). The readings were taken using a microplate spectrophotometer (SpectraMax® Plus 384 Microplate Reader by Molecular).

The other enzymatic markers were performed via automated clinical biochemistry (Urit 8021A MHLab) by absorbance photometry and Ionogram by indirect potentiometry (WAMA WE300) using commercial kits (BioTecnica®, Varginha/MG, Brazil).

We quantify the following enzymatic markers: total creatine kinase (CK-NAC), creatine kinase-MB (CK-MB), and lactate dehydrogenase (LDH).

2.3 Arterial and venous catheterization

After anesthesia (ketamine-xylazine; 75mg + 15 mg/kg, i.p.) two cannulae (P10 -Micro-Renathane connected to P50 cannulae (ClearTygon)) were inserted; one into the femoral vein for drug application, and one in the femoral artery for blood pressure register. At the end of the surgery, it was administered anti-inflammatory analgesic (Meloxican, 0.4 mg/kg, i.v.) and antibiotic (Enrofloxacin 5 mg/kg, i.v.) for recovery. The cannulas were heparinized (250 units/mL) after the surgery and the animals were kept in individual boxes for recovery. After

48 hours of recovery (26–29), the protocol for assessment of response to stress and sympathetic vascular tone modulation was carried.

2.4 Arterial pressure recording

The experiments were performed after one hour of room adaptation, baseline arterial pressure records were performed for 30 minutes before the protocol. After the baseline recording, intravenous injection of the hexamethonium or stress evaluation was performed according to the protocol described below. Experiments were carried out between 10:00 and 15:00 in conscious free-moving animals.

The arterial cannula was connected to a pressure transducer (MLT0670, ADInstruments, Dunedin, New Zealand) which was connected to a recording system (Insight, Ribeirão Preto, Brazil) that uses the DI 158 System for signal acquisition and analog-to-digital signal conversion (WinDaq lite, DataQinstruments, USA). Recordings were sampled at 1000Hz.

Data were pre-analyzed using specific Advanced CODAS software (Data Q instruments, United States of America, https://www.dataq.com/products/windaq/, paid software) to objectively identify stable periods of recording and exclusion of erratic signals. The baseline values of systolic arterial pressure (SAP), diastolic (DAP), mean (MAP) and pulse interval (PI) were transferred to a spreadsheet for analysis (Microsoft Excel, Redmond, WA, EUA) over 10 minutes of recording under stable conditions. Heart rate (HR) was calculated from the pulse interval (ms), considering the formula (60000 / PI in ms).

2.5 Cardiovascular variability and baroreflex sensitivity

In the basal blood pressure recording it was estimated the autonomic impact on baseline MAP was evaluated in the frequency domain through the analysis of MAP variability, using CardioSeries 2.4 Software (USP - Ribeirão Preto, http://www.danielpenteado.com/, freely available). The power spectra were calculated using a fast Fourier transform (FFT) as previously described (7,30–32). Briefly, the Beat-by-beat series obtained from pulsatile arterial pressure recordings were converted to data points every 100 ms using cubic spline interpolation (10 Hz). The interpolated series were divided into half-overlapping sequential sets of 512 data points (51.2 s). The low-frequency band of the MAP (LF-MAP: 0.2 - 0.75 Hz of the spectrum) was used as an estimate of vascular sympathetic activity (7,33,34), the low-frequency band of PI (LF-PI: 0.2-0.75 Hz of the spectrum) estimates the cardiac sympathetic activity. The estimate of cardiac parasympathetic activity with the high-frequency band of the PI (HF-PI: 0.75–3 Hz of the spectrum) and the sympathovagal balance was estimated by the LF/HF ratio in the PI spectrum (7,34,35).

The spontaneous cardiac baroreflex sensitivity was evaluated through the sequence method using CardioSeries 2.4 Software (http://www.danielpenteado.com/, freely available), which calculates the spontaneous baroreflex through the angle of the linear regression between MAP and PI, with a delay of 3 heartbeats. Sequences with at least 3 intervals were considered only if the correlation of the coefficients was very high (>0.85). The spontaneous cardiac baroreflex sensitivity was estimated by the average of significant angles (31). The baroreflex effectiveness index (BEI) was calculated by the ratio between the total number of PI/MAP sequences and the total number of MAP ramps (36).

2.6 Vascular sympathetic tone

After baseline blood pressure recordings, it was administered the ganglion blocker, hexamethonium (10 mg/kg; i.v.; Sigma-Aldrich, San Luis, MO, USA) (28,37). The delta of MAP was calculated to assess the sympathetic contribution to blood pressure.

2.7 Cardiovascular response to behavioral stress test

In a separate cohort of animals, after the baseline blood pressure recordings, the animals were placed in a cylindrical plexiglass restrainer (Insight, Ribeirão Preto, Brazil) for five minutes. The rat was returned to their boxes after the test (38,39). The delta of MAP and PI was calculated by subtracting the values obtained during the stress test and recovery from the basal blood pressure to assess the response.

2.8 Neuroinflammation in the medulla and forebrain

The animals were anesthetized with sodium thiopental (40mg/kg; ip) and perfused transcardially with 300 ml of 0.1M phosphate-buffered saline (PBS, pH 7.4) followed by 300 ml of 4% paraformaldehyde (PFA; Labsynth, São Paulo, Brazil) in PBS. Brains were collected, fixed for 4h in 4% PFA solution, and stored in PBS with 20% sucrose (Labsynth, São Paulo, Brazil) at 4°C for at least seven days (40,41). Four serial sets (30 µm) of the paraventricular nucleus of hypothalamus (PVN), commissural nucleus of the solitary tract (cNTS), intermediate nucleus of the solitary tract (iNTS) and rostral ventrolateral medulla (RVLM) were sectioned in cryostat (Leica, CM1800) and the sections were stored in cryoprotectant at -20°C until the immunohistochemistry.

The sections were rinsed in 0.1 M PBS (3 x 5 min) and following incubated at 15 min with a blocking solution of 10% normal goat serum (NGS, Vector Laboratories, California, USA) and 0.3% Trition X-100 (Sigma-Aldrich, San Luis, MO, USA) in 0.1 M PBS (40). After rinse in PBS (3 x 10 min), the sections were incubated in a rabbit anti-Iba-1 primary antibody (1:3.000; Wako Chemicals, Richmond, VA, USA) and mouse anti-GFAP (1:400, Chemicon International, Temecula, CA, USA) in PBS with 1% NGS and 0.3% Trition X-100 for 48 hours at 4°C. After the incubation, the sections were rinsed in PBS (3 x 10 min) and incubated with Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 594 goat anti-mouse (both 1:500; Life Technologies and Invitrogen, California, USA) for 1 hour (42). Following, sections were rinsed in PBS (3 x 10 min), were mounted on gelatin slides, which were air-dry and after coverslipped with fluorescent mountant (Abcam, Cambridge, UK) (41).

For the RVLM, the sections went through two protocols. Sections were incubated in a rabbit anti-Iba-1 primary antibody (1:3.000; Wako Chemicals, Richmond, VA, USA) and mouse monoclonal tyrosine hydroxylase (TH) primary antibody (1:200, ImmunoStar Inc., Hudson, USA) in PBS with 1% normal horse serum (NHS, Sigma-Aldrich, San Luis, MO, USA) and 0.3% Trition X-100 for 48 hours at 4°C. After the incubation, the sections were rinsed in PBS and incubated with biotinylated horse anti-mouse IgG (1:500; Vector Laboratories, California, USA) for 1 hour. The sections were rinsed in PBS, then incubated with Alexa Fluor 594 Streptavidin conjugated and Alexa Fluor 488 donkey anti-rabbit (both 1:500; Life Technologies, California, USA) for 1 h. After the incubation, the sections were rinsed with PBS and mounted as described above.

Other sections of the RVLM were incubated with mouse anti-GFAP (1:400, Chemicon International, Temecula, CA, USA) and goat anti-choline acetyltransferase (ChAT) in PBS with 1% NGS and 0.3% Trition X-100 for 48 hours at 4°C. After the incubation, the sections were rinsed in PBS, and following they were incubated with biotinylated horse anti-mouse IgG

(1:500; Vector Laboratories, California, USA) for 1 hour. The sections were rinsed in PBS and incubated with Alexa Fluor 594 Streptavidin conjugated and Alexa Fluor 488 donkey anti-goat (both 1:500; Life Technologies, California, USA) for 1 h. After the incubation, the sections were rinsed with PBS and mounted as described above. The sections were captured at 10x magnification with a fluorescence microscope (Leica DM5500 B, Wetzlar, Hessen, Germany).

2.9 Echocardiogram, and electrocardiogram

One cohort of animals was anesthetized with thiopental (40mg/kg; ip) and submitted to transthoracic echocardiography using the ultrasound equipment model MYLAB 25 Gold (Esaote S.pA., Genova, Italy), equipped with a Neonatal Transducer Model PA023 (4- 11MHZ).

The animals were positioned in an adapted stretcher in the supine position. The first image in two-dimensional mode was obtained in the right parasternal longitudinal section (long axis) to visualize chambers 4C and 5C. In M mode, it was positioned perpendicular to the interventricular septum and posterior wall of the left ventricle at the level of the papillary muscle and in M mode images were obtained for wall thickness and chamber dimensions.

The internal diameters of the left ventricle in systole (LVDs) and diastole (LVDd), the thickness of the interventricular septum in systole (IVSs) and diastole (IVSd), and the thickness of the LV posterior wall in systole (PWs) and diastole (PWd) were analyzed in diastole and systole. The ejection fraction (EF) and shortening fraction (SF) were calculated using the TEICHHOLZ method using automated algorithms in the equipment software. All parameters were measured at least three times per animal. All parameters were regulated by cardiac mass, except the FS and EF (43), the other results that were not regulated by cardiac mass are available in Supplementary Table 3.

After the echocardiogram, the electrocardiogram (ECG) signal was captured. The electrodes were inserted transcutaneously in four limbs and the signals from the six leads were recorded continuously for at least 10 minutes. It was recorded bipolar peripheral leads (D1, D2 and D3) or unipolar peripheral leads (aVR, aVL and aVF). Data were analyzed in derivation D2. For signal acquisition, we used the equipment INcardio Agile Duo Acquisition System Version 2.9 (Florianopolis, Brazil) and for analysis, we used the INpulse software (Florianopolis, Brazil).

The parameters were determined for each ECG waveform analysis: RR interval, P duration and amplitude, PR interval and segment, QRS interval and amplitude, QT interval, QTc interval, ST segment and T duration and amplitude. The intervals were calculated using algorithms in the equipment's software.

2.10 mRNA expression of IL6 and TNF-α in the heart

Samples of the left ventricle were collected. Tissues were frozen in nitrogen and subsequently stored at -80°C. Total RNA was extracted using TRIzol™ (Invitrogen®) following the manufacturer's instructions. Total RNA concentration was measured spectrophotometrically at 260 nm (NanoDrop ND 1000 NanoDrop Technologies, Wilmington, DE). For cDNA synthesis 2 µg of RNA was converted into cDNA using a high-capacity cDNA reverse transcription kit (Promega US, Madison, WI, USA), and samples were run using an AcessQuick™ Master Mix (Promega US, Madison, WI, USA).

RT-PCR was performed using the Promega Access RT-PCR System (Promega US, Madison, WI, USA). The first strand of cDNA synthesis was obtained by one cycle of 5 min at 25°C,

followed by one cycle of 1 min at 42 °C and one cycle of 15 min at 70 °C. The sample was then sequentially thermocycled at 95°C for 2 min, then 40 cycles for 15 sec at 95 °C, and 40 cycles for 1 min at 60 °C. The expression patterns of genes of interest were normalized to constitutively expressed beta-actin and the primers used were described (Supplementary Table 4). The $2^{-\Delta CT}$ method was used for the relative quantification analysis and data were expressed in arbitrary units (AU) (44,45).

2.11 Statistical analysis

The results are presented as mean ± standard error of the mean (SEM). The data distribution was performed before the statistical analysis (Shapiro-Wilk test). Results are considered significant when $P < 0.05$. GraphPad Prism 8.4.3 (GraphPad software, La Jolla, CA, USA, (https://www.graphpad.com/scientific-software/prism/) was used for statistical analysis. The comparison between the groups was performed using the Student's T test using diet as a factor. The two-way ANOVA was used considering time and diet as independent factors.

3 Results

3.1 Biochemical and electrolyte analyses

LP animals showed a statistically significant increase in glycemia (LP: 111 ± 2.92 vs. NP: 102 ± 1.28 mg/dl, p=0.019) and triglyceridemia (LP: 130 ± 14.67 vs. NP: 91 ± 10.55 mg/dl, p=0.041), compared with NP animals. The total cholesterol was similar between the groups (Table 1).

The markers of the tissue damage, the LP animals showed an increase in CK-NAC (LP: 6837 ± 366 vs. NP: 5896 ± 250 UI/L, p=0.039), CK-MB (LP: 1229 ± 84 vs. NP: 1019 ± 53 UI/L, p=0.039) and LDH (LP: 1795 ± 123 vs. NP: 1454 ± 76 UI/L, p=0.022) compared to controls animals (Table 2).

3.2 Basal arterial pressure

The LP animals showed an increase in SAP (LP: 143 ± 2.56 vs. NP: 127 ± 2.65 mmHg, p = 0.001; Fig. 1A), DAP (LP: 92 ± 0.73 vs. NP: 87 ± 1.69 mmHg, p = 0.048; Fig. 1B) and MAP (LP: 111 ± 1.27 vs. NP: 102 ± 0.91 mmHg, p = 0.0003; Fig. 1C) compared with NP animals. The HR was similar between the groups.

3.3 Cardiovascular variability and baroreflex sensitivity

Table 3 shows the results of short-term arterial pressure variability evaluation during baseline. LP animals had an increase in the LF-MAP (LP: 4.46 ± 0.20 vs. NP: 3.33 ± 0.24 $mmHg^2$; p = 0.002; Table 3) and LF-PI bands (LP: 3.04 ± 0.31 vs. NP: 2.01 ± 0.28 ms^2; p = 0.029; Table 3), estimating 3an increase in vascular and cardiac sympathetic activity. The LP animals showed an increased LF / HF ratio (LP: 0.25 ± 0.030 vs. NP: 0.15 ± 0.016 ms^2; p = 0.007; Table 3) estimating a contribution of the sympathetic activity and showed a decrease in HF-PI band (LP: 10 ± 0.85 vs. NP: 16 ± 1.65 ms^2; p = 0.005; Table 3), estimating a reduction in parasympathetic activity. The LP animals also showed an increase in Gain (LP: 5.92 ± 0.25 vs. NP: 8.22 ± 0.67;

p = 0.005; Table 3), pointing to an increased baroreflex sensitivity, without difference in BEI compared to NP animals.

3.4 Vascular sympathetic tone

After blocking the autonomic nervous system with hexamethonium, the LP group showed a greater depressor response in MAP (LP: -36 ± 4.27 vs. NP: -20 ± 5.06 mmHg; p = 0.029; Fig. 2A) compared with NP animals.

3.5 Cardiovascular response to behavioral stress test

During the five minutes of the stress test, the LP animals showed a greater increase in MAP (LP: 14 ± 1.06 vs. NP: 10 ± 0.96 mmHg; p = 0.012; Fig. 3A) and PI response (LP: -16 ± 3.49 vs. NP: -5 ± 3.60 ms; p = 0.041; Fig. 3B) compared with control animals. During the 5 min recovery of the stress test, LP animals showed a greater reduction in PI (LP: -17 ± 3.96 vs. NP: -6 ± 2.94 ms; p = 0.036; Fig. 3D), with no difference in MAP response between the groups.

3.6 Neuroinflammation in the medulla and forebrain

In LP group, the number of Iba-1 positive cells was increased in PVN (LP: 42 ± 2.45 vs. NP: 33 ± 2.17 cell number/section; p = 0.029; Fig. 4A), RVLM (LP: 84 ± 3.74 vs. NP: 56 ± 2.14 cell number/section; p = 0.0002; Fig. 4B) and iNTS (LP: 33 ± 2.31 vs. NP: 23 ± 1.69 cell number/section; p = 0.009; Fig. 4D) with no difference in cNTS compared to NP animals.
The immunoreactivity for GFAP in LP group was increased in cNTS (LP: 22 ± 1.57 vs. NP: 18 ± 1.02 gray values/section; p = 0.046; Fig. 5B) e iNTS (LP: 15 ± 0.94 vs. NP: 11 ± 0.19 gray values/section; p = 0.004; Fig. 5C) without difference in PVN between the groups.

3.7 Echocardiogram, and electrocardiogram

In the echocardiogram, the LP animals showed an increase in IVSd thickness (LP: 1.06 ± 0.024 vs. NP: 0.98 ± 0.025 mm; p = 0.040; Fig. 6A), LVDd (LP: 3.44 ± 0.066 vs. NP: 3.16 ± 0.086 mm; p = 0.012; Fig. 6B), and PWd (LP: 1.16 ± 0.027 vs. NP: 1.06 ± 0.021 mm; p = 0.008; Fig. 6C) in diastole compared with NP animals. In the systole, the LP animals showed an increase in IVSs thickness (LP: 1.58 ± 0.041 vs. NP: 1.34 ± 0.052 mm; p = 0.001; Fig. 6D), LVDs (LP: 1.97 ± 0.060 vs. NP: 1.76 ± 0.064 mm; p = 0.023; Fig. 6E), and PWs (LP: 1.49 ± 0.032 vs. NP: 1.33 ± 0.025 mm; p = 0.0009; Fig. 6F) compared with control animals. The SF was decreased in LP animals (LP: 41 ± 0.81 vs. NP: 45 ± 1.74; p = 0.042; Fig. 6G) with no difference in EF between the groups.

In the electrocardiogram, the LP animals had an increase in R-R interval (LP: 185 ± 3.13 vs. NP: 174 ± 3.70 s; p = 0.035; Table 4) compared with NP rats. No significant changes were observed in P duration, PR interval, PR segment, QRS interval, QT interval, QTc interval, ST segment and T duration between the groups.

The analyses of wave amplitude showed that the LP animals have an increase in T-wave (LP: 0.106 ± 0.008 vs. NP: 0.078 ± 0.007 mV; p = 0.030; Table 4) with no difference in P-wave, Q-wave, R-wave, and S-wave between the groups.

3.8 mRNA expression of IL6 and TNF-α in the heart

The IL6 and TNF-α levels were increased in LP compared with NP animals (LP: 3.13 ± 0.81 vs. NP: 0.98 ± 0.41 AU; p = 0.043; and LP: 5.13 ± 1.46 vs. NP: 0.94 ± 0.23 AU; p = 0.047; respectively; Fig. 7A).

4 Discussion

This work demonstrates that the hypertensive adult rats programmed by a caloric-protein restriction in the peripubertal phase show neuroinflammation and cardiac inflammation, which may mediate the hypersympathetic activity and cardiac dysfunction. The present meta-inflammation may mediate the autonomic dysfunction, leading to increased blood pressure and increased pressure response to stress, as well as cardiac remodeling and dysfunction (46–48), as evidenced by: 1) neuroinflammation in areas of blood pressure regulation; 2) increase in cardiac and vascular sympathetic activity in basal blood pressure recording; 3) increased pressure response to restraint test; 4) increased cardiac inflammation; 5) increased circulatory marker of the cardiac lesion; 6) increase in T-wave amplitude; and 7) greater diameter and wall thickness of the left ventricle. This is the first study that points to meta-inflammation as a potential mediator of adult neurogenic hypertension and cardiac remodeling programmed by a caloric-protein peri-pubertal insult.

Previously we have shown that LP animals have sympathetic hyperactivity (7). The present study explores dipper this issue showing an increased blood pressure response to stress test and an increase in neuroinflammation in the RVLM, pointed by the increase in the number of Iba-1 positive cells in LP animals. The RVLM neurons modulate the peripheral blood pressure, as its activation results in an increase in sympathetic efferent activity to the heart, arteries, and kidneys elevating the blood pressure (49). The microglia, as in situ macrophages in the brain, can regulate neuroimmune responses in several situations (50). It has been shown that microglial cells can be activated in hypertension, especially these cells were found in the cardiovascular nucleus of blood pressure control (PVN and RVLM), resulting in peripheral sympathetic hyperactivity (20) and pathological myocardial remodeling (51). The present data demonstrate an increase in microglia activation in RVLM, which may modulate sympathetic hyperactivity resulting in an increased pressure response to stress test and to the ganglia blocker, the hexamethonium, as well as an increase in cardiac sympathetic activity in basal blood pressure. Considering these results, we hypothesize that accentuated activity of RVLM neurons is the primary cause of essential hypertension and may contribute to cardiovascular remodeling. Present LP animals exhibit baroreflex dysfunction accompanied by neuroinflammation in the NTS, as evidenced by a reduction in spontaneous baroreflex sensitivity, increased GFAP immunoreactivity in the cNTS and iNTS, and a higher number of Iba-1 positive cells in the iNTS. The baroreceptor sensory inputs send bursts to sensory neurons in the NTS, which results in the inhibition of sympathetic activity and excitation of vagal tone to reverse the increase in blood pressure (52). Baroreflex sensitivity is important to evaluating the NTS function in blood pressure regulation (53). It has been shown that systemic inflammation induces an increase in the level of proinflammatory cytokines in the NTS (54), which can promote damage in the NTS neuron's activity and lead to baroreflex desensitization (55). The present data points to long-term baroreflex desensitization mediated by NTS neuroinflammation induced by peripubertal caloric-protein restriction.

In the LP animals, we also show an increased microglia number in PVN and RVLM, which points to neuroinflammation in these areas related to cardiovascular control mediated by the autonomic nervous system. It has been shown that the activated microglia in the PVN can increase the pro-inflammatory cytokines, directly increasing the activity of sympathetic neurons (19); into the RVLM, leading to increases in sympathetic outflow (56) and increasing blood pressure. In addition to this, the increase in circulating angiotensin 2 can activate the cardiovascular-responsive areas in the CVOs resulting in the generation and transmission of hypertensive signals to the PVN (57). It has been shown that the brain renin angiotensin system activation, via peripheral angiotensin signaling to CVOs, may induce a neuroinflammation (58). Previously, we have shown an increase in the renin-angiotensin system activity with sympathoexcitation in adult hypertensive LP animals programmed by peripubertal caloric-protein restriction (6,7). In other models with severe hypertension with sympathoexcitation, the microglia in PVN are more activated and the administration of anti-inflammatory agents, such as IL-10 or minocycline, into the brain can suppress autonomic nervous system activity and reduce elevated blood pressure (19). In this context, we may suggest that hyperactivation of the renin angiotensin system may mediate the present neuroinflammation, which could be a key mechanism contributing to the development of hypertension in LP animals. Further studies need to be performed to explore dipper this issue.

The increase in heart inflammation may lead to cardiac abnormalities in the electrical conduction system (59). Previously, we have shown that the animals programmed by caloric-protein restriction in peripubertal life have an increase in blood pressure (6,7) with cardiovascular hypertrophy and an increase in collagen deposition in the heart (7). The present study explores further cardiac function and remodeling. The echocardiogram shows compromised cardiac function and remodeling. The echocardiogram points to an increased diameter of the left ventricle, with a low capacity to reduce the size between diastole and systole in LP animals. This architecture and pattern of function may mediate a future cardiac failure. Previous studies showed an overworking heart just after peripubertal exposure to protein restriction (43) and a cardiovascular dysfunction in adulthood, characterized by previously reported hypertrophy and collagen deposition (7) and the present cardiac remodeling. This pattern may result in a severe increase in the risk of heart failure during aging (60). Furthermore, the electrocardiogram showed that LP animals have a lower heart rate and an increased amplitude of T wave, which estimates ventricular repolarization (43). Increased T-wave amplitude may point to ventricular hypertrophy (61,62), which accords with the findings in the echocardiogram. In line with these findings, LP animals showed elevated levels of a cardiac marker of lesion, as the serum enzymes LDH, CK, and CK-MB, reflect injury and impairment of cardiac function (63).

The mechanism leading to cardiac dysfunction and remodeling may depend on the increase in heart inflammation. The present LP animals show an increase in TNF-α and IL6 mRNA expression in the left ventricle. It has been shown that early life exposure during pregnancy and lactation to protein restriction induces a low-grade systemic inflammation characterized by elevated levels of pro-inflammatory cytokines such as TNF-α, with a tendency for increased IL-6 levels observed just after the insult (64). This pattern would contribute to the development of cardiovascular diseases, however, from our knowledge, this issue was not explored before in the DOHaD context of protein restriction. In this context, the present findings are the first to show long-term heart inflammation associated with cardiac abnormalities induced by early life exposure to a caloric-protein restriction. Studies with spontaneously hypertensive rats showed

an increase in IL-1β, IL-6, and TNF-α in the serum and left ventricle in adult life (22,23). Furthermore, the proinflammatory cytokines have been linked to pathological cardiac hypertrophy (65), which is observed in the present LP animals. Many studies over the last two decades have shown that IL-1β, IL-6, and TNF-α are intimately linked to hypertension, cardiac fibrosis, pathological cardiac remodeling, and cardiac hypertrophy (66–68). Furthermore, the present RVLM neuroinflammation may also be contributing to cardiac remodeling. It has been shown that the PLX3397 (pexidartinib), used to deplete central microglia, administrated via intragastric or intracerebroventricular protected cardiac function and inhibited cardiac remodeling after myocardial infarction, with attenuation of cardiac inflammation, cardiomyocyte apoptosis, cardiac fibrosis, and electrical remodeling, possibly through the modulation of cardiac sympathetic remodeling that was regulated by the neuroimmune response (51).

Previous studies in the peripubertal protein restriction model have shown that these animals develop metabolic syndrome in adulthood, characterized by increased adipose tissue, hypertriglyceridemia, hyperglycemia, and insulin resistance(6,10). Concomitant with the metabolic syndrome in this study we demonstrated an increased neuroinflammation and cardiac inflammation. This inflammation is strongly associated with the onset of metabolic syndrome (25) in adulthood, thereby providing evidence of the meta-inflammation in this model. Other studies have shown a low-grade inflammation just after exposure to protein restriction in other critical phases of development (64,69). In this context, we may suggest that meta-inflammation can establish the connection between neurogenic hypertension and cardiovascular dysfunction in the models programmed to nutrition insults in the peripubertal period. Furthermore, the present model of metabolic syndrome shows insulin resistance, which has been considered a key element mediating the cluster of risk factors enrolled in this syndrome (70). It has been pointed that inflammation plays a causal role in the development of insulin resistance, which emphasizes the central role of meta-inflammation in the physiopathology of metabolic syndrome and its risk factors, as hypertension, type 2 diabetes, increased visceral adiposity and dyslipidemia (71).

5 Conclusion

In summary, the present study shows that peripubertal caloric-protein restriction induces meta-inflammation which may be mediating the hypersympathetic activity, neurogenic hypertension, and cardiac remodeling and dysfunction observed in adulthood. Furthermore, the increased activity of the renin-angiotensin system, previously reported in the present model, may be enrolled in the present meta-inflammation contributing to the hypersympathetic activity, hypertension and heart dysfunction, however more studies need to be performed to better explore the correlation between these mechanisms in the present model. This study provides a better understanding of the mechanisms involved in the long-lasting health condition induced by peri-pubertal caloric-protein restriction and open new insights about other systems enrolled in the physiopathology of hypertension observed in the present metabolic syndrome programmed in prepuberty. Additionally, these findings point to potential pharmacological interventions, such as the regulation of meta-inflammation, to control the hypertension induced by peripubertal insult. Furthermore, it is important to consider new public health interventions in peri-pubertal life to prevent or decrease long-term hypertension and cardiovascular dysfunction potentially induced by malnutrition.

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Tables

Table 1. Effects of dietary protein restriction in the peri-pubertal period on biochemical parameters.

Biochemical parameters	NP	LP	P value
Blood glucose (mg/dL)	102 ± 1.28	111 ± 2.92	0.019*
Triglycerides (mg/dL)	91 ± 10.55	130 ± 14.67	0.041*
Total cholesterol (mg/dL)	96 ± 7.36	91 ± 6.06	0.624

NP: Normal protein diet; LP: Low protein diet; n = 7 to 14 animals per group. Values expressed as mean ± SEM. * p <0.05, indicates statistical significance (Student's T test).

Table 2. Effects of dietary protein restriction in the peri-pubertal period on electrolytes and tissue damage markers.

Parameters	NP	LP	P value
Tissue damage			
CK-NAC (UI/L)	5896 ± 250	6837 ± 366	0.039*
CK-MB (UI/L)	1019 ± 53	1229 ± 84	0.039*
LDH (UI/L)	1454 ± 76	1795 ± 123	0.022*

CK-NAC: Total Creatine Phosphokinase-NAC; CK-MB: Creatine phosphokinase-MB; LDH: and lactate dehydrogenase; NP: Normal protein diet; LP: Low protein diet; n = 8 to 14 animals per group. Values expressed as mean ± SEM. * p <0.05, indicates statistical significance (Student's T test).

Table 3. Cardiovascular variability and baroreflex sensitivity.

Parameters	NP	LP	P value
Cardiovascular variability			
LF MAP ($mmHg^2$)	3.33 ± 0.24	4.46 ± 0.20	0.002**
LF PI (ms^2)	2.01 ± 0.28	3.04 ± 0.31	0.029*
HF PI (ms^2)	16 ± 1.65	10 ± 0.85	0.005**
LF / HF PI	0.15 ± 0.016	0.25 ± 0.030	0.007**
Baroreflex sensitivity			
BEI	0.07 ± 0.012	0.06 ± 0.017	0.662
Gain (spontaneous baroreflex)	8.22 ± 0.67	5.92 ± 0.25	0.005**

LF-MAP: low-frequency band of the mean blood pressure; LF-PI: low-frequency band of pulse interval; HF-PI: high-frequency band of the pulse interval; LF/HF: sympathovagal balance in the pulse interval; BEI: baroreflex effectiveness index; NP: Normal protein diet; LP: Low protein diet; n = 8 to 10 animals per group. Values expressed as mean ± SEM. ** p <0.01 and * p <0.05, indicate statistical significance (Student's T test).

Table 4. Effects of dietary protein restriction in peri-pubertal period in electrocardiogram in anesthetized animals.

Parameters	NP	LP	P value
Wave-length			
P Duration (ms)	34 ± 0.617	35 ± 0.482	0.069
R-R Interval (s)	174 ± 3.70	185 ± 3.13	0.035*
PR Interval (ms)	44 ± 0.666	44 ± 0.954	0.842
PR Segment (ms)	9.55 ± 0.555	9.71 ± 0.624	0.992
QRS Interval (ms)	37 ± 0.534	37 ± 0.450	0.948
QT Interval (ms)	62 ± 1.176	61 ± 0.443	0.859
QTc Interval (ms)	77 ± 1.359	75 ± 0.677	0.233
ST segment (ms)	2 ± 0.000	2.14 ± 0.142	0.999
T Duration (ms)	22 ± 1.296	22 ± 0.521	0.821
Wave amplitude			
P wave amplitude (mV)	0.076 ± 0.002	0.091 ± 0.005	0.071
Q wave amplitude (mV)	-0.030 ± 0.002	-0.027 ± 0.003	0.577
R wave amplitude (mV)	0.345 ± 0.019	0.349 ± 0.023	0.899
S wave amplitude (mV)	0.017 ± 0.011	0.038 ± 0.012	0.259
T wave amplitude (mV)	0.078 ± 0.007	0.106 ± 0.008	0.030*

NP: Normal protein diet; LP: Low protein diet; n = 7 to 14 animals per group. Values expressed as mean ± SEM. * p <0.05, indicates statistical significance (Student's T test).

Figures

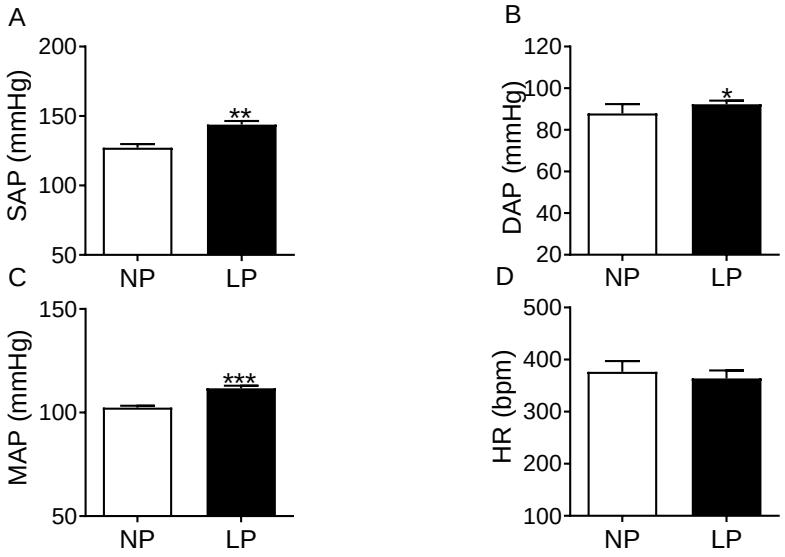


Fig. 1. Basal arterial pressure. systolic arterial pressure (A), diastolic arterial pressure (B), mean arterial pressure (C), and heart rate (D). N = 5 - 10 animals per group. NP, normal protein diet; LP, low protein diet. Values expressed as mean ± SEM. *** p <0.001, ** p <0.01, and * p <0.05, indicate statistical significance (Student's T test).

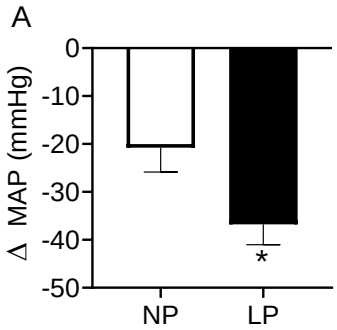
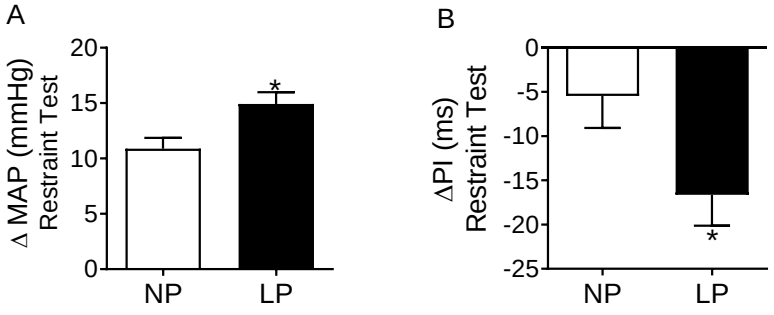


Fig. 2. Blood pressure response after hexamethonium. change in mean arterial pressure in response to hexamethonium (A). N = 7 - 10 animals per group. NP, normal protein diet; LP, low protein diet. Values expressed as mean ± SEM. * p <0.05, indicates statistical significance (Student's T test).



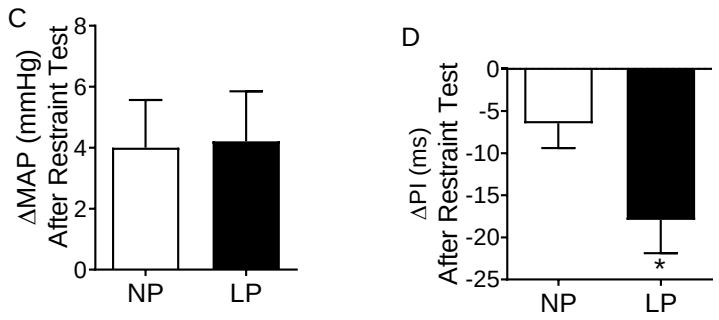


Fig. 3. Response during and before the restraint test. change in mean arterial pressure in response to restraint test (A), change in pulse interval in response to restraint test (B), change in mean arterial pressure in response after the restraint test (C), change in pulse interval in response after the restraint test (D). N = 9 - 12 animals per group. NP, normal protein diet; LP, low protein diet. Values expressed as mean ± SEM. * p <0.05, indicates statistical significance (Student's T test).

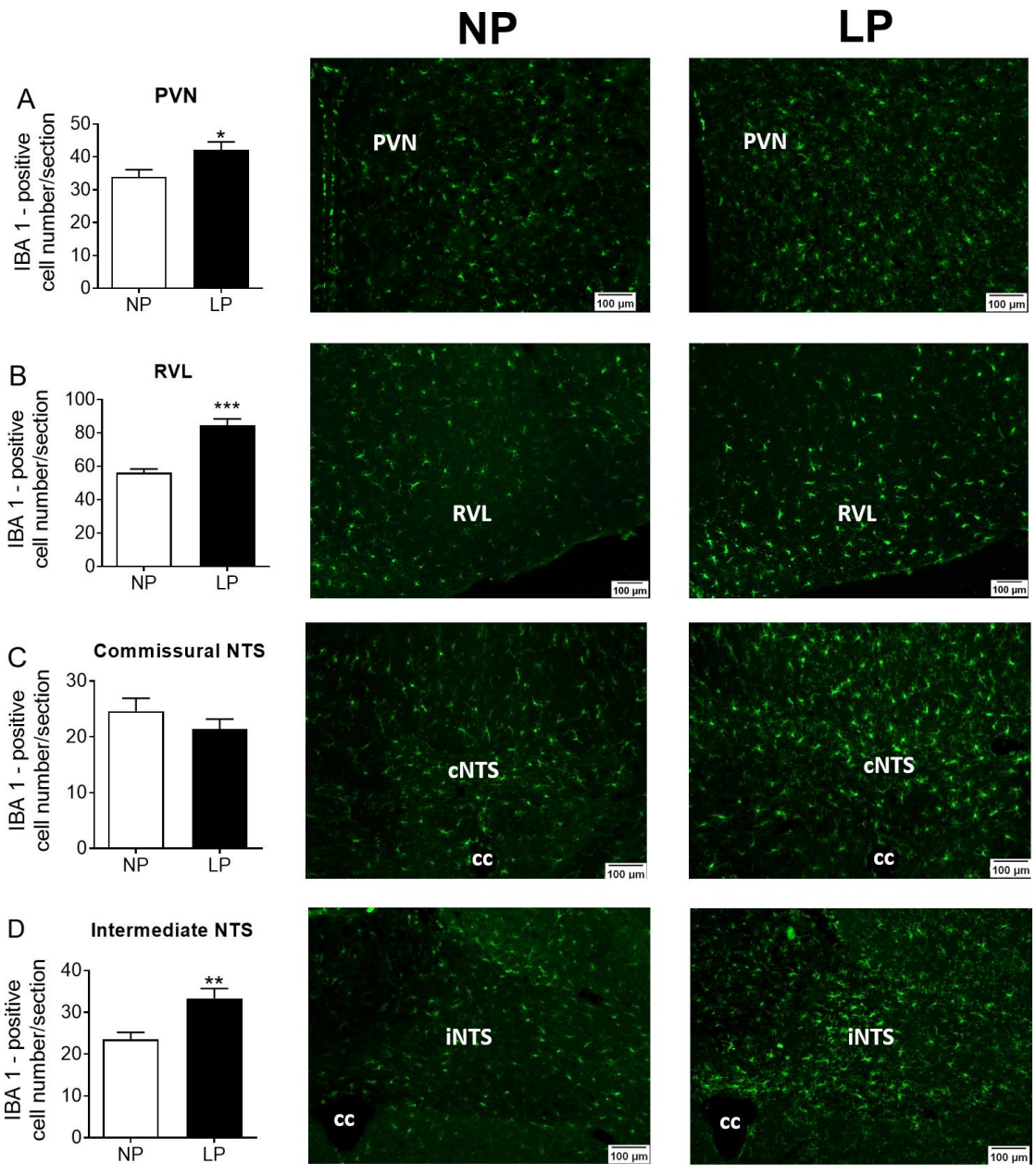


Fig. 4. Iba-1 immunoreactivity. Iba-1 immunoreactivity in the paraventricular nucleus of hypothalamus (PVN) (A), Iba-1 immunoreactivity in the rostral ventrolateral medulla (RVLM) (B), Iba-1 immunoreactivity in the commissural nucleus of the solitary tract (cNTS) (C), Iba-1 immunoreactivity in the intermediate nucleus of the solitary tract (iNTS) (D). N = 5 - 7 animals per group. Scale bar = 100 µm. NP, normal protein diet; LP, low protein diet. Values expressed as mean ± SEM. *** p <0.001, ** p <0.01, and * p <0.05, indicate statistical significance (Student's T test).

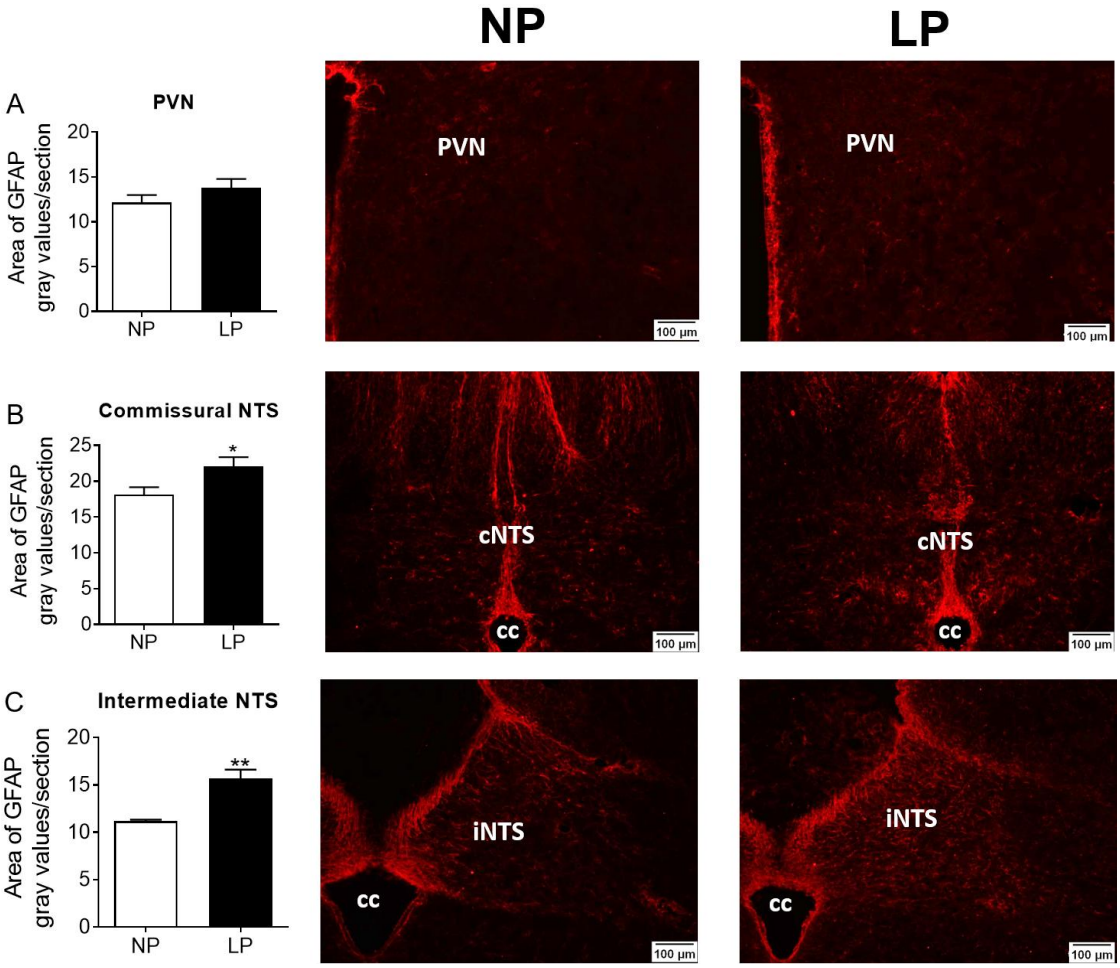
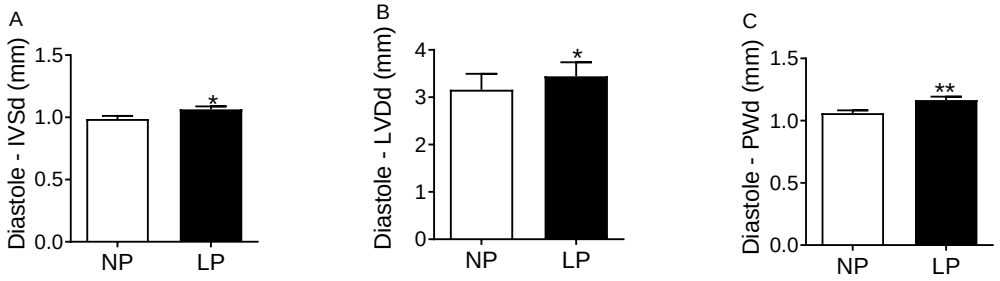


Fig. 5. GFAP immunoreactivity. GFAP immunoreactivity in the commissural nucleus of the solitary tract (cNTS) (A), GFAP immunoreactivity in the intermediate nucleus of the solitary tract (iNTS) (B), and GFAP immunoreactivity in the paraventricular nucleus of the hypothalamus (PVN) (C). N = 5 - 7 animals per group. Scale bar = 100 µm. NP, normal protein diet; LP, low protein diet. Values expressed as mean ± SEM. ** p <0.01, and * p <0.05, indicate statistical significance (Student's T test).



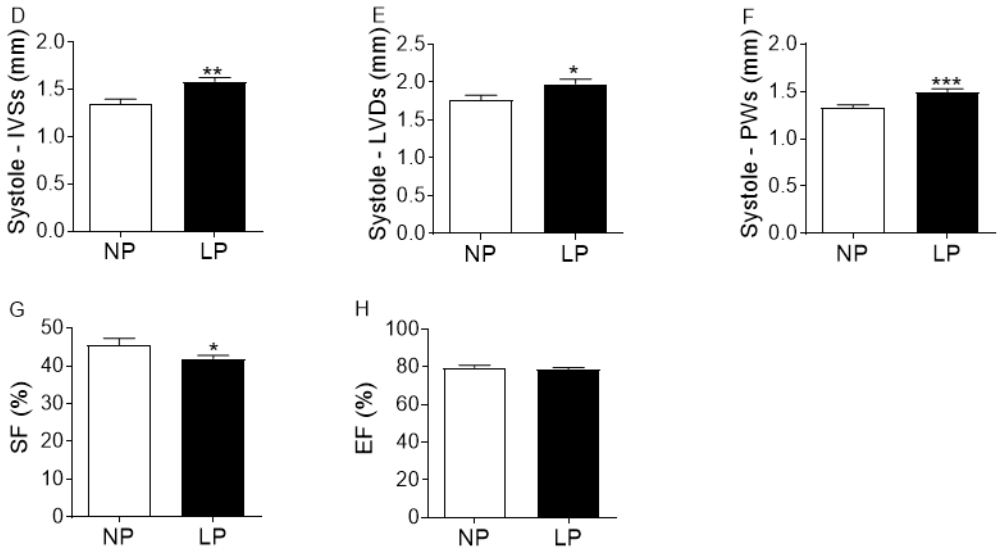


Fig. 6. Echocardiogram normalized by heart weight. diastolic thickness of interventricular septum (IVSd) (A), estimation of left ventricle internal diameter in diastole (LVDd) (B), left ventricular posterior wall during diastole (PWd) (C), systole thickness of interventricular septum (IVSs) (D), estimation of left ventricle internal diameter in systole (LVDs) (E), left ventricular posterior wall during systole (PWs) (F), shortening fraction (G), ejection fraction (H). N = 11 - 20 animals per group. NP, normal protein diet; LP, low protein diet. Values expressed as mean ± SEM. *** p <0.001, ** p <0.01, and * p <0.05, indicate statistical significance (Student's T test).

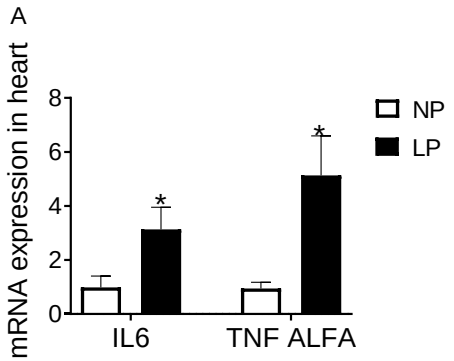


Fig. 7. Inflammation mRNA expression in the heart. mRNA expression of IL6 e TNF-α in the heart (A), n = 6 to 10 animals per group. NP, normal protein diet; LP, low protein diet. Values expressed as mean ± SEM. * p <0.05, indicates statistical significance (Student's T test).

Supplementary material

Supplementary Table 1. Composition of the isocaloric low-protein and normal-protein diets.

Diet components	Normal protein (20.5 %)	Low protein (4.0 %)
Sucrose	12.72	20.00
Corn starch	52.75	64.25
Casein (88% protein)	23.33	4.55
Mix of mineral salts	3.20	3.20
Mix of vitamins	1.60	1.60
Soybean oil	4.80	4.80
Fish oil	1.60	1.60
Total (g)	100.0	100.0

Supplementary Table 2. Effects of dietary protein restriction in peri-pubertal period on biometric parameters

Biometric parameters	NP	LP	P value
Body weight (g)	421 ± 4.80	379 ± 3.96	0.0001***
Naso-anal length (cm)	23.75 ± 0.23	22.78 ± 0.25	0.022*
Retroperitoneal fat pad (g/100g bw)	1.71 ± 0.10	2.02 ± 0.09	0.042*
Mesenteric fat pad (g/100g bw)	0.85 ± 0.04	0.74 ± 0.03	0.072
Heart (g)	1.38 ± 0.031	1.28 ± 0.030	0.039*
Heart (g/100g bw)	0.32 ± 0.0072	0.35 ± 0.0076	0.012*

NP: Normal protein diet; LP: Low protein diet; n = 6 to 28 animals per group. Values expressed as mean ± SEM. *** $p < 0.001$, and * $p < 0.05$, indicate statistical significance (Student's T test).

Supplementary Table 3. Effects of dietary protein restriction in the peri-pubertal period on echocardiogram parameters

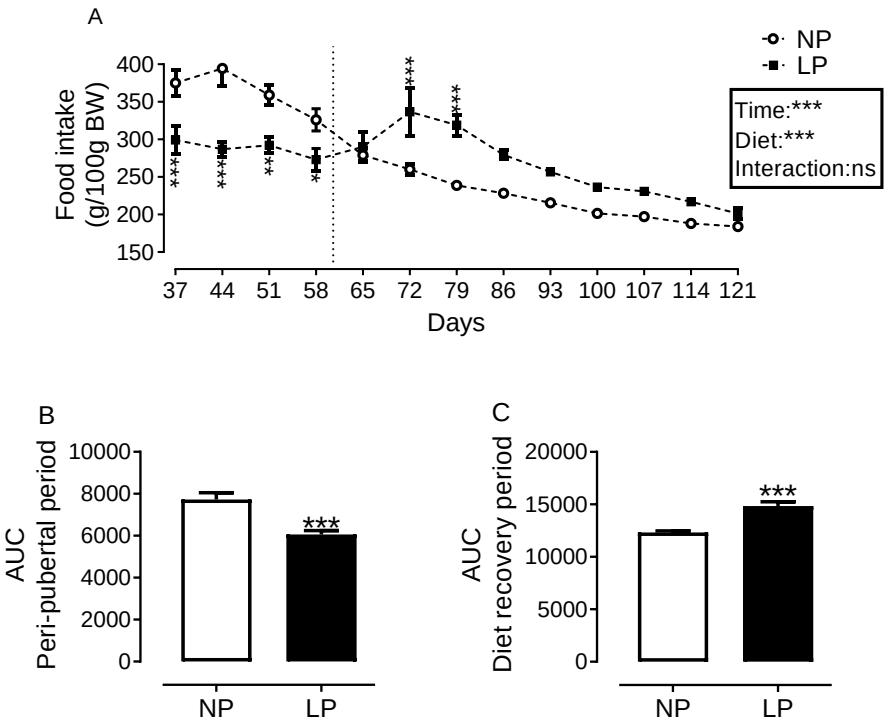
Echocardiogram parameters	NP	LP	P value
Systole			
Interventricular septum (mm)	2.93 ± 0.11	2.94 ± 0.09	0.948

LV diameter (mm)	3.84 ± 0.112	3.83 ± 0.117	0.970
LV posterior wall(mm)	2.99 ± 0.11	2.89 ± 0.04	0.347
Diastole			
Interventricular septum (mm)	2.17 ± 0.06	2.06 ± 0.03	0.131
LV diameter (mm)	6.88 ± 0.15	6.67 ± 0.12	0.288
LV posterior wall(mm)	2.31 ± 0.053	2.25 ± 0.050	0.428

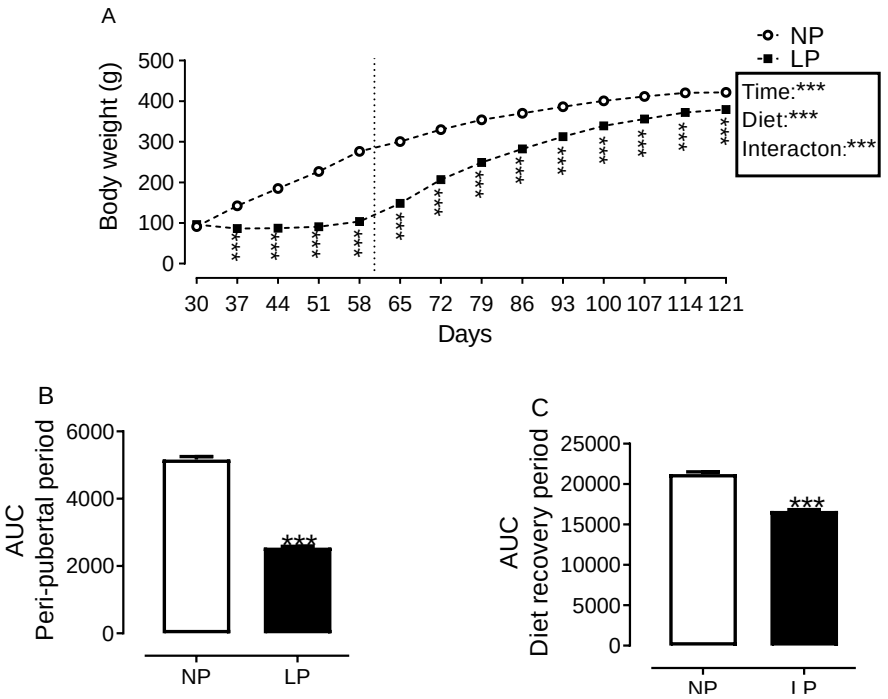
LV: Left ventricle; NP: Normal protein diet; LP: Low protein diet; n = 15 to 20 animals per group. Values expressed as mean ± SEM.

Supplementary Table 4. List of primers used for RT-PCR.

Primers	Sequence (5'-3') Forward	Sequence (5'-3') Reverse
Beta actin	5' GTCGTACCACTGGCATTGTG 3'	5' CTCTCAGCTGTGGTGGTGAA 3'
Tnfα	5' ATGGGCTCCCTCTCATCAG 3'	5' GCTTGGTGGTTTGCTACGA3'
IL-6	5' CATTCTGTCTCGAGCCCAC3'	5' GCTGGAAGTCTCTTGCGGA 3'



Supplementary Fig. 1. Food intake. Food consumption (A), AUC food consumption during the peri-pubertal period (B), AUC food intake during the dietary recovery period (C), n = 26 to 28 animals per group. NP, normal protein diet; LP, low protein diet. Values are presented as mean ± SEM. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$, indicate statistical significance (Student's T test or Two-way ANOVA).



Supplementary Fig. 2. Body weight. Body weight through the experimental protocol. (A), AUC of body weight during the peri-pubertal period (B), AUC of body weight during the dietary recovery period (C). n = 26 to 28 animals per group. NP, normal protein diet; LP, low protein diet. Values are presented as mean ± SEM. *** $p < 0.001$, indicates statistical significance (Student's T test or Two-way ANOVA).

CAPÍTULO III

Conclusões

No presente estudo a exposição na peripuberdade a restrição proteica induziu disfunções tanto no coração como no sistema renina angiotensina com uma maior inflamação na periferia e no encéfalo na vida adulta da ratos *Wistar*, mostrando que:

1. A puberdade é uma fase crítica do desenvolvimento, suscetível à programação por meio de insultos dietéticos, o que pode levar a alterações no funcionamento do sistema cardiovascular e do sistema renina-angiotensina na vida adulta.

2. O aumento da atividade do sistema renina-angiotensina pode estar mediando a hipertensão nesse modelo de restrição proteica na peripuberdade

3. A restrição calórica-proteica na peripuberdade induz meta-inflamação que pode estar mediando o aumento da atividade simpática, hipertensão neurogênica e remodelação e disfunção cardíaca.

O aumento da atividade do sistema renina-angiotensina, pode estar implicado na hiperatividade simpática e meta-inflamação observada. Por sua vez, a meta-inflamação pode estar implicada na hiperatividade simpática e aumento da atividade do sistema renina angiotensina. Este ciclo pode estar contribuindo para a hipertensão e disfunção cardíaca. Com base nesses resultados, potenciais intervenções farmacológicas podem ser exploradas neste modelo, embora ainda necessitem de uma investigação mais detalhada.

Perspectivas futuras

Considerando os resultados advindos desse estudo, estudar a implicação de intervenções farmacológicos na peripuberdade, visando a redução da inflamação sistêmica e da neuroinflamação podem contribuir para confirmar o mecanismo fisiopatológico da hipertensão neurogênica e da hiperatividade simpática.

A realização da análise morfológica da micróglia pode contribuir para reconhecer qual o estado de ativação da micróglia, essenciais para o desenvolvimento de novas intervenções contribuindo para o potencial translacional dos achados experimentais do presente estudo.

A realização da dosagem das citocinas inflamatórias no sangue durante a peripuberdade e na vida adulta pode auxiliar a confirmar a inflamação sistêmica de baixo grau nesse modelo, contribuindo para identificação do mecanismo fisiopatológicas e a descoberta de estratégias de intervenção na peripuberdade.

A exploração dos mecanismos de regulação epigenética desde a peripuberdade pode contribuir para descobrir quais mudanças epigenéticas podem estar influenciando na expressão de genes relacionados a processos metabólicos, inflamatórios e cardiovasculares. Auxiliando na identificação dos biomarcadores precoces para doenças associadas a insultos nutricionais.