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RESTRIÇÃO PROTEICA MATERNA INDUZ HIPERATIVIDADE SIMPÁTICO-RESPIRATÓRIA E ALTERA A EXPRESSÃO DE GENES METABÓLICOS HEPÁTICOS E MUSCULARES NA PROLE DE RATOS: ESTUDO DOS MECANISMOS FISIOLÓGICOS E MOLECULARES

RECIFE
2015

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Tese apresentada ao Programa de Pós-Graduação em Neuropsiquiatria e Ciências do Comportamento do Centro de Ciências da Saúde da Universidade Federal de Pernambuco, como requisito à obtenção do título de Doutor em Neurociências.

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RESUMO

Investigamos se alterações simpático-respiratórias e de quimiossensibilidade poderiam estar envolvidas no desenvolvimento de hipertensão arterial (HA) em ratos expostos à restrição proteica durante a gestação e lactação. Pesquisamos também o perfil de expressão de genes no músculo e fígado envolvidos com metabolismo de glicose e ácidos graxos. Ratas Wistar foram alimentadas com dieta normoproteica (grupo controle) ou com dieta hipoproteica (grupo experimental) durante a gestação e lactação. Após o desmame, a prole de ratos machos (grupo NP provenientes de mães controle e grupo HP provenientes de mães experimentais) foram alimentados com dieta padrão e os estudos funcionais realizados ao 30º e 90º dia de vida. A pressão arterial (PA) foi aferida de forma direta pelo implante de cânula na artéria femoral e a ventilação foi mensurada por pletismografia. O registro da atividade do nervo simpático torácico e frênico foi realizado na preparação coração tronco cerebral isolados. Quimiorreceptores periféricos (QRP) foram ativados por KCN (0.04 µg/Kg, 100uL/rato). QRP foram removidos para investigar o seu papel na gênese da hipertensão arterial. Músculo esquelético, fígado e tronco encefálico foram coletados. Ensaios de expressão gênica foram realizados por PCR-RT e proteica por Western Blotting. No tronco encefálico os níveis de peroxidação lipídica (avaliado pelos níveis de malondialdeído-MDA) e atividade das enzimas antioxidantes SOD e catalase foram avaliados. Os resultados mostraram que aos 30 dias de vida, ratos HP apresentaram PA semelhante ao grupo NP. Contudo, o grupo HP exibiu, nesta idade, aumento na atividade simpático-respiratória associada com maior sensibilidade de quimiorreceptores periféricos. Aos 90 dias, ratos HP apresentaram HA, associado, em parte, com um aumento de peroxidação lipídica e redução na atividade de SOD e catalase na área ventral do tronco encefálico. A remoção dos QRP atenuou a PA e a atividade simpática em ratos HP. Aos 30 dias de vida, observamos uma redução na expressão gênica da PDK4, βHAD no músculo sóleo e de HK2, PFK, CS e PGC1a no músculo EDL de ratos HP. A expressão gênica de G6Pase, βHAD e CS foi aumentada no fígado de ratos HP. Verificamos também, um aumento na expressão proteica de HK2 e uma redução na expressão proteica de PDK4 e βHAD no músculo sóleo de ratos HP. Analisando o músculo EDL, o grupo HP exibiu uma redução na expressão proteica de HK2 e PFK, mas sem alteração na expressão de βHAD e CS. Aos 90 dias de vida, ratos HP exibiram redução na expressão gênica de PDK4 no músculo sóleo e EDL. Em nível hepático, o grupo HP apresentou maior expressão gênica de FAS e baixa expressão gênica de PGC1a associado com redução na expressão proteica de βHAD. Os resultados indicam a) hiperatividade simpática e respiratória associada com maior

sensibilidade de quimiorreceptores periféricos no início da vida; b) remoção de QRP atenua HA em ratos HP; c) HA na vida adulta de ratos HP está associada a um desequilíbrio oxidativo; d) a expressão de genes metabólicos envolvidos no metabolismo da glicose e dos ácidos graxos está alterada no fígado e músculo de ratos HP.

Palavras-chave: Desnutrição. Hipertensão. Células quimiorreceptoras. Metabolismo energético.

ABSTRACT

In the present study, we investigated whether the sympatho-respiratory and chemosensory alterations can be involved in the development of arterial hypertension in protein-restricted offspring rats during gestation and lactation. In addition, we researched in these animals, the profile of gene expression in the muscle and liver involved with glucose and fatty acids metabolism. Females Wistar rats received normoproteic diet (17% of protein, control group, NP) or low protein diet (8% of protein, experimental group, LP) during gestation and lactation. After the weaning, the male offspring received standard diet and the functional studies were performed at 30 and 90-d-old. The straight register of arterial blood pressure was measured by femoral artery catheter and the ventilation was recorded by pletismography. The recording of thoracic sympathetic and phrenic nerve was realized by isolated brainstem-heart preparation (WHBP). The peripheral chemoreceptors were activated by KCN (0.04%, 100 μL/rat). In addition, it was realized removal of the peripheral chemoreceptors to investigate its role in the genesis of arterial hypertension. Gene expression for Hk2, PFK, PDK4, CPT1b, bHAD, CS, PGC1a, PEPCK, G6Pase, FAS, SOD1 and 2, CAT, GPX, Grin1, Gria1, e Grm1 were performed by the RT-PCR. In addition, protein expression was performed by Western blotting assay. In the brainstem were investigated the lipid peroxidation levels (by malondialdehyde-MDA assay) and enzymatic activity for superoxide dismutase and catalase. At 30 d-old Lp rats presented similar arterial pressure in comparison to Np rats. Although, Lp group exhibited, in this age, increase in the sympathetic-respiratory activity linked to higher peripheral chemosensitivity. At 90d-old, Lp rats presented hypertension, which in part, was linked to enhanced lipid peroxidation and decreased anti-oxidant enzymatic activity into ventral medulla. The carotid body removal attenuated arterial pressure and sympathetic actitiy in protein-restricted rats during pregnancy and lactation. At 30 d-old, the studies performed in skeletal muscle and liver, demonstrated a reduced mRNA expression for PDK4, βHAD in soleus muscle and the HK2, PFK, CS and PGC1a in EDL muscle of Lp group. In addition, we noted that mRNA expression for G6Pase, βHAS and Cs were enhanced in the Lp liver. We found that protein expression of HK2 was increased in Lp soleus and PDK and βHAD reduced in comparison to Np group. In EDL, protein expression for HK2 and PFJ were reduced in Lp rats. At 90 d-old, mRNA for PDK4 was dow-expressed in soleus and EDL from Lp rats. In the liver, mRNA for FAS was up-expressed in Lp rats and mRNA PGC1a and β down-regulated. The results indicates that: i) sympathetic and respiratory hiperactivity linked to with higher peripheral chemosensitivity in early life can contribute to development

of arterial hypertension in adult life from Lp rats. ii) Carotid body removal attenuates the hypertension in rats submitted the perinatal protein restricted. iii) Arterial hypertension in Lp adult life is associated with oxidative disbalance. iv) metabolic genes expression involved in glucose and fatty acids metabolism are altered, at short and long-term, in liver and muscle of rats submitted the perinatal protein restricted.

Key-word: Malnutrition, Hypertension. Quimioreceptors cells. Energetic metabolism.

LISTA DE ABREVIATURAS E SIGLAS

HAS	Hipertensão Arterial Sistêmica
CEUA	Comitê de ética em experimentação animal
AIN	Do inglês, American Institute of Nutrition
DM2	Diabetes Mellitus tipo 2
NP	Do inglês, Normal protein group
LP	Do inglês, Low protein group
MAP	Do inglês, mean arterial pressure
HR	Do inglês Heart rate
DANT	Doenças e Agravos Não-Transmissíveis
RVLM	Região Rostral Lateral da medula
CVLM	Região Caudal Lateral da Medula
SOD	Superóxido Dismutase
CAT	Catalase
GPX	Glutationa peroxidase
MDA	Malondialdeído
Grin1	Do inglês, Glutamatergic receptor ionotropic NMDA 1
Gria1	Do inglês, Glutamatergic receptor ionotropic AMPA 1
Grm1	Do inglês, Glutamatergic receptor metabotropic 1
HK2	Hexoquinase 2
PFKM	Fosfofrutoquinase muscular
PDK4	Piruvato desidrogenase quinase 4
CPT1	Carnitina Palmitoil Transferase 1
βHAD	beta Hidroxi-Acil-Coa Desidrogenase
CS	Citrato Sintase
PGC1a	Do inglês, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PEPCK	Fosfoenolpiruvato C quinase
G6Pase	Glicose 6 fosfatase
FAS	Do inglês, Fatty acid syhntase

CBD	Do inglês, Carotid Body Denervation
LF	Do inglês, Low frequency
HF	Do inglês, High frequency
tSN	Do inglês, thoracic sympathetic nerve
PN	Do inglês, phrenic nerve
KCN	Cianeto de potássio
RT-PCR	Reação de polimerase em cadeia em tempo real

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Sumário

1. Apresentação

Nas últimas décadas, tem sido registrado um aumento exponencial na prevalência doenças e agravos não transmissíveis (DANT), a exemplo da obesidade, resistência à insulina e hipertensão arterial no mundo (Hedner *et al.*, 2012). Portanto, compreender os fatores predisponentes é sem dúvida um desafio, em virtude da etiologia multifatorial destas patologias.

Interessantemente, estudos experimentais e epidemiológicos demonstraram que eventos adversos, a exemplo da restrição proteica, *in útero* ou durante o período perinatal (gestação, lactação e primeira infância) consiste em um fator de risco para o desenvolvimento de DANTs na vida adulta dos filhos (Langley-Evans *et al.*, 1999; Johansson *et al.*, 2007; De Brito Alves, Nogueira, *et al.*, 2014a).

A relação entre insultos nutricionais durante o período perinatal e o desenvolvimento de DANTs na vida adulta dos indivíduos tem sido fundamentada no conceito de "fenótipo econômico" ou "thrifty phenotype", na qual o feto é capaz de se adaptar e sobreviver em um ambiente de pobre nutrição (Barker *et al.*, 1990; Hales e Barker, 1992). A curto prazo, estas adaptações fenotípicas ajudam à sobrevivência, mas a longo-prazo, suscetibilizam o organismo ao aparecimento de DANTs (Barker, 1999). Apesar destes estudos mostrando que a desnutrição durante o período perinatal predispõe DANTs, pouco se sabe a respeito dos mecanismos que contribuem para seu desenvolvimento.

Tem sido proposto que um aumento na atividade nervosa simpática e respiratória associada a uma maior sensibilidade de quimiorreceptores pode predispor o desenvolvimento de hipertensão (Guyenet *et al.*, 1989; Smith *et al.*, 1991; Guyenet, 2006; Zoccal *et al.*, 2009; Costa-Silva *et al.*, 2010; Paton *et al.*, 2013).

Em adição, modificações na expressão de enzimas envolvidas no metabolismo de ácidos-graxos e glucose, sobretudo no músculo esquelético, fígado e tecido adiposo, parecem contribuir para o desenvolvimento de resistência à insulina e *diabetes mellitus* tipo 2 (Ozanne *et al.*, 1996; Muhlhausler *et al.*, 2009; Da Silva Aragao *et al.*, 2014; Ozanne, 2015).

Neste sentido, esse trabalho procurou investigar a relação entre possíveis alterações nos mecanismos de controle simpático e respiratório associado com o desenvolvimento da hipertensão arterial em ratos submetidos à desnutrição proteica perinatal.

Uma outra via de investigação do presente trabalho, foi verificar, a curto e longo prazo, possíveis alterações em genes e proteínas chaves envolvidas no metabolismo de ácidos-

graxos e glicose no músculo esquelético e no fígado de ratos expostos à restrição proteica durante a gestação e lactação.

2. Revisão de Literatura

Nas últimas décadas tem sido registrado um aumento exponencial da prevalência de hipertensão arterial, afetando quase 1 bilhão de pessoas no mundo, além de ser reconhecida como a principal causa de morbi-mortalidade da população (Hedner *et al.*, 2012). No entanto, a causa subjacente da hipertensão arterial torna-se difícil de se identificar devido sua natureza multifatorial, na qual fatores genéticos e ambientais podem predispor o seu desenvolvimento (Hedner *et al.*, 2012).

Tem sido reportado que eventos adversos experimentados no útero ou durante o período perinatal (gestação, lactação e primeira infância) podem afetar o desenvolvimento de sistemas fisiológicos e aumentar a predisposição de hipertensão arterial e doenças metabólicas na vida adulta (Barker *et al.*, 1990; Gluckman e Hanson, 2004). O fenômeno biológico subjacente a essa associação é denominado "plasticidade fenotípica", e se refere à capacidade de um fenótipo associado a um único genótipo para produzir variações no desenvolvimento dos organismos em resposta às circunstâncias ambientais, em termos de comportamento, morfologia e/ou fisiologia (West-Eberhard, 1986). Uma das variações mais bem documentadas no estudo da plasticidade fenotípica é a nutrição. A falta ou o aumento do aporte nutricional durante períodos críticos do desenvolvimento podem resultar em alterações permanentes na estrutura e função de órgãos e predispor o desenvolvimento de doenças não comunicantes (West-Eberhard, 1986; Fidalgo *et al.*, 2012; Leandro, Da Silva Ribeiro, *et al.*, 2012; De Brito Alves, Nogueira, *et al.*, 2014a).

A associação entre vida perinatal e aparecimento de doenças no adulto é chamada de *Thrifty Phenotype Hypothesis* e foi enunciada por Hales e Barker (1992) (Barker e Martyn, 1992). Esta hipótese propõe que estímulos ou insultos (por exemplo, desnutrição, fumo, antígenos, drogas e álcool) ocorridos durante a gestação e lactação induzem adaptações fisiológicas e metabólica. A curto prazo, estas adaptações fenotípicas ajudam à sobrevivência, mas a longo-prazo, suscetibilizam o organismo ao aparecimento de distúrbios metabólicos (Hales e Barker, 1992). Nos últimos anos, esta hipótese tem sido amplamente utilizada para interpretar as associações entre o peso ao nascer e o risco tardio de doenças cardiovasculares

Uma das primeiras evidências epidemiológicas associando desnutrição durante a gestação e maior predisposição de desenvolvimento de doenças cardiovasculares na vida adulta dos filhos foi relatada em indivíduos que nasceram durante a fome holandesa na II guerra mundial entre os anos de janeiro de 1945 e março de 1946 (Stein *et al.*, 2006).

Outros estudos epidemiológicos têm corroborado com essa hipótese. Por exemplo, estudo realizado em Enugu (n=1339) durante a guerra civil nigeriana (1967-1970) demonstrou que a exposição à fome durante a fase fetal e infantil foi associada com elevada pressão sistólica (+ 7 mmHg; $p < 0,001$) e diastólica (mmHg + 5; $p < 0,001$) em indivíduos adultos (Hult *et al.*, 2010).

Um estudo de coorte retrospectivo com 12.065 indivíduos nascidos durante a grande fome chinesa entre os anos de 1957-1964 demonstrou que o risco de hipertensão na vida adulta foi 1.36 vezes maior nos indivíduos expostos à fome durante o primeiro trimestre (intervalos de confiança de 95% - 1,03-1,79) de gestação e 1.83 vezes (OR 1.61-2.08) maior entre aqueles expostos à fome durante a infância (Wang *et al.*, 2012).

Recentemente um outro estudo realizado com judeus europeus nascidos entre 1940-1945 em países nazistas durante o holocausto revelou que a prevalência de hipertensão foi significativamente maior (62.9% vs. 43%, P=0.003) quando comparados aos indivíduos nascidos em Israel da mesma descendência e mesma idade (Bercovich *et al.*, 2014).

Diante desses achados, torna-se evidente que a desnutrição durante períodos críticos de desenvolvimento apresenta uma relação causal com maior chance de desenvolvimento de hipertensão arterial na vida adulta dos indivíduos.

Em modelos experimentais, restrição proteica materna durante a gestação e lactação é um dos modelos animais mais estudados da hipótese de origem desenvolvimentista de doenças metabólicas. Por exemplo, ratos adultos submetidos à desnutrição proteica (5 a 8% de proteína) perinatal desenvolvem hipertensão arterial na vida adulta (Langley-Evans *et al.*, 1999; Costa-Silva *et al.*, 2009; De Brito Alves, Nogueira, *et al.*, 2014a). Prévios estudos têm demonstrado que o aumento de pressão arterial nesse modelo de desnutrição proteica está associado relacionado a um menor número de néfrons e glomérulos, bem como devido uma hiperatividade do sistema renina-angiotensina-aldosterona e arginina-vasopressina (Langley-Evans *et al.*, 1999; Cornock *et al.*, 2010)

Além disso, outros estudos demonstraram que a desnutrição durante o período fetal é um fator de risco para o desenvolvimento de hipertensão na prole, devido o contato excessivo do feto com glicocorticoides (Seckl *et al.*, 2000). Estudos com modelos experimentais de desnutrição tem observado uma redução da atividade da 11β-hidroxiesteróide desidrogenase (11βHD), enzima esta, indispensável na proteção do feto à elevações de glicocorticoides através da conversão de corticosterona em 11-dehidrocorticosterona (Benediktsson *et al.*, 1993; Gwathmey *et al.*, 2011). Uma vez que a atividade dessa enzima encontra-se reduzida, maior é o contato do feto com os glicocorticóides, podendo levar a uma maior captação de

sódio e cálcio no músculo cardíaco, além de prejudicar o desenvolvimento da glândula adrenal, por meio da supressão da secreção do hormônio adrenocortocotropina (ACTH). Isso resultaria em um prejuízo na comunicação do eixo hipotálamo-hipófise-adrenal e maior predisposição a quadros de elevação de pressão na prole (Benediktsson *et al.*, 1993). Essas comprovações iniciais servem de sustento para pesquisas mais atuais, as quais sustentam a hipótese de que a desnutrição na gestação altera a atividade da enzima 11βHD e favorece o aumento do contato do feto com glicocorticoides (Belkacemi *et al.*, 2011; Jones *et al.*, 2012).

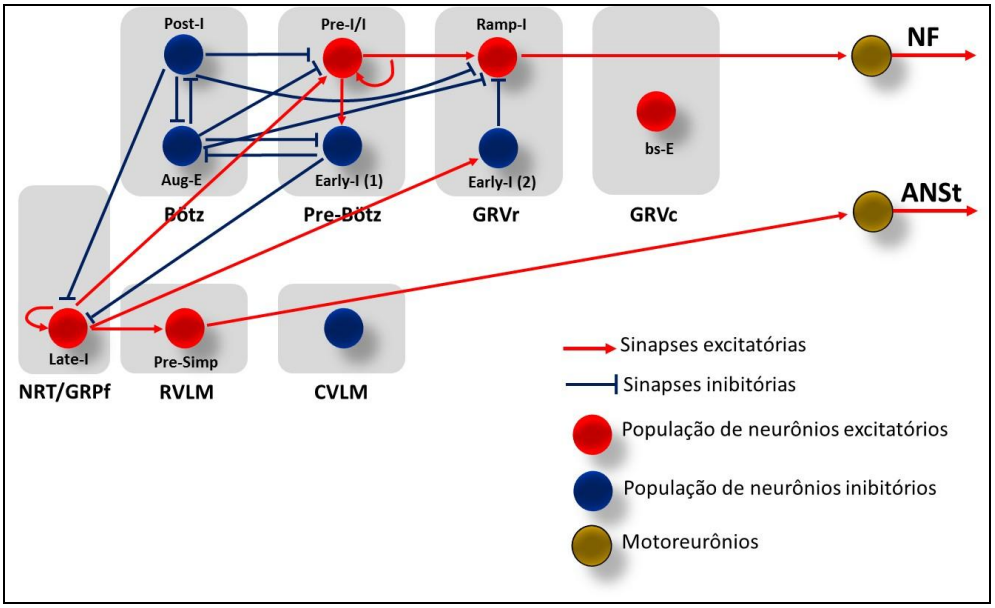
Apesar destes resultados, ainda não estão claros quais os mecanismos periféricos e centrais podem contribuir para o desenvolvimento da hipertensão arterial. Nessa perspectiva, nosso laboratório tem se dedicado tem sugerido que disfunções do sistema respiratório e simpático contribuem para a instalação da hipertensão arterial nestes indivíduos provenientes de mães que sofreram desnutrição proteica perinatal.

Recentemente estudos do nosso laboratório demonstrou que ratos submetidos à desnutrição proteica (8% de proteína) durante a gestação e lactação desenvolvem hipertensão arterial na vida adulta, a qual foi associada a mudanças no ritmo respiratório, maior sensibilidade de quimiorreceptores periféricos e aumento de atividade simpática (Barros *et al.*, 2014; De Brito Alves, Nogueira, Cavalcanti-Neto, *et al.*, 2014; De Brito Alves, Nogueira, *et al.*, 2014a). Esses achados propõem uma nova abordagem acerca dos mecanismos subjacentes ao desenvolvimento e hipertensão arterial em nosso modelo de desnutrição.

A respiração é um complexo processo pelo qual o oxigênio (O_2) é captado do ar ambiente e é levado até as células para a obtenção da energia necessária para as funções vitais do organismo (Biscoe e Sampson, 1967). Evidências prévias têm demonstrado que neurônios respiratórios localizados na coluna respiratória ventral (área ventral do tronco cerebral), são essenciais para geração do ritmo respiratório (Bianchi *et al.*, 1995; Smith *et al.*, 2007). As atividades destes neurônios são responsáveis pela orquestração do ciclo respiratório, sendo classificados em: pré-inspiratório (pre-inspiratory, pre-I), com atividade antes do início da inspiração; inspiratório inicial (early inspiratory, early-I), com atividade na fase inicial da inspiração; inspiratório final (late inspiratory, late-I), com atividade na fase final da inspiração; pós-inspiratório (post-inspiratory, post-I), como o próprio nome já diz, apresenta atividade na fase pós-inspiratória; e expiratório final (late expiratory, late-E e os de ampliação expiratória, Aug-E), com atividade na fase final da expiração (Bianchi *et al.*, 1995; Moraes *et al.*, 2013).

A CRV é subdividida no sentido rostro-caudal em 4 regiões funcionalmente distintas: i) complexo Bötzinger (BötC), região que contém principalmente neurônios expiratórios e

pós-inspiratórios (Post-I e Aug-E); ii) complexo pré-Bötzinger (pré- BötC), grupo de inter-neurônios pre-I/I e early-I considerados como essenciais para a geração da atividade inspiratória; iii) porção rostral do grupo respiratório ventral (rVRG), o qual contém neurônios pré-motores late-I; e iv) porção caudal do grupo respiratório ventral (cVRG), o qual contém neurônios pré-motores que se projetam para os núcleos da medula espinhal (**Figura 1**) (Ezure, 1990; Smith *et al.*, 1991; Bianchi *et al.*, 1995; Smith *et al.*, 2007).



Adaptado de Molkov e Zoccal et al., 2011.

Figura 01. Modelo esquemático mostrando a interação entre diferentes populações de neurônios respiratórios no tronco encefálico envolvidos no controle da atividade simpática e respiratória. **Abreviações dos compartimentos do tronco encefálico:** Complexo Bötzinger (Bötz), complexo Pré-Bötzinger (Pré-Bötz), Grupo respiratório ventral rostral (GRVr), Grupo respiratório ventral causal (GRVc), Núcleo Retrotrapezóide e Grupo Respiratório Parafacial (NRT/GRPF), Região Rostral ventrolateral da medula (RVLM), Região Caudal Ventro-Lateral da Medula (CVLM). **Abreviações das populações de neurônios:** Pós-Inspiratório (Post-I), Aumentação expiratória (Aug-E), Pré-Inspiratório (Pré-I), Inspiratório (I), Inspiratório inicial 1 (early– I), Inspiratório inicial 2 (early– 2), Rampa-Inspiratório (Ramp-I), Finalizador inspiratório (late-I), pré-simpático (pré-simp) e bulbo espinal pré-motor E (bs-E).

Na CRV, sobretudo na região rostral ventral lateral do bulbo (RVLM) estão também localizados neurônios geradores da atividade simpática (Guyenet *et al.*, 1990; Dampney, 1994; Moraes *et al.*, 2013). O RVLM consiste de um grupo de neurônios, estendendo-se desde a extremidade caudal do núcleo facial à medula ventrolateral caudal (CVLM) com uma extensão anteroposterior de 700 µm em ratos adultos (Dobbins e Feldman, 1994; Moraes *et al.*, 2011). No RVLM há dois grandes grupos de neurônios pré-simpáticos glutamatérgicos:

um expressa todas as enzimas necessárias para produção de adrenalina (grupo C1), neurônios esses apresentam atividade de marca-passo intrínseca, dependente de corrente sódio-fósforo (I_{NaP}); e o outro corresponde à neurônios não-catecolaminérgicos (Reis *et al.*, 1989; Ruggiero *et al.*, 1994; Stornetta *et al.*, 2002; Moraes *et al.*, 2011; Moraes *et al.*, 2013).

Neurônios inspiratórios e expiratórios do tronco cerebral, principalmente os da coluna respiratória ventral (CRV), os da ponte e o do núcleo retrotrapezóide (RTN) podem estabelecer interações excitatórias ou inibitórias com neurônios do RVLM (Guyenet *et al.*, 1990; Miyawaki *et al.*, 1995; Moraes, Dias, *et al.*, 2012). Isso demonstra que neurônios pré-simpáticos glutamatérgicos da RVLM são modulados pela atividade respiratória (Mcallen, 1987; Haselton e Guyenet, 1989). Em outras palavras, há um acoplamento entre neurônios simpáticos e respiratórios, no qual a atividade simpática de repouso apresenta aumentos fásicos predominantemente durante a inspiração (Dick *et al.*, 2004; Zoccal e Machado, 2011).

A primeira evidência de possível conexão entre neurônios envolvidos na regulação do sistema respiratório e autonômico foi descrito por Traube e Hering (Moraes, Zoccal, *et al.*, 2012). Traube observou a ocorrência de grandes ondas de pressão arterial após a interrupção da ventilação artificial em cães e gatos vagotomizados, enquanto Hering notou que cada uma dessas ondas de pressão arterial, mais tarde chamadas de ondas Traube – Hering, correlacionava-se com um movimento dos músculos respiratórios. Além disso, Traube também observou que animais com o nervo vago intacto apresentava aumento de frequência cardíaca durante a inspiração, a qual era associada com uma diminuição da atividade vagal para o coração. Portanto, o Traube foi o primeiro a sugerir que inervações simpáticas e parassimpáticas para o sistema cardiovascular são significativamente influenciadas pela atividade respiratória. Estudos posteriores por Adrian e colaboradores confirmaram, por registros diretos do nervo simpático, que a atividade simpática exibe oscilações rítmicas associadas à atividade respiratória (Adrian *et al.*, 1932).

Além do importante papel fisiológico e benéfico do acoplamento simpático-respiratório, tem sido demonstrado que a hiperativação dos mecanismos envolvidos com esse acoplamento é relevante para o desenvolvimento de hiperatividade simpática (Guyenet *et al.*, 1990; Zoccal *et al.*, 2009) .

A hiperativação simpática é caracterizada por um aumento na intensidade e na frequência das despolarizações elétricas do nervo simpático e também por um aumento nos níveis plasmáticos de catecolaminas promovendo constrição dos vasos sanguíneos periféricos, aumento na resistência vascular periférica e, consequentemente aumento nos níveis basais da pressão arterial (Malpas, 1998; Zoccal *et al.*, 2009)

Portanto, mudanças no acoplamento simpático-respiratório tornou-se relevante no cenário dos possíveis mecanismos chaves subjacentes ao desenvolvimento da hipertensão arterial. A primeira evidência experimental de um alterado acoplamento simpático-respiratório associado com hipertensão arterial foi demonstrado por Czyzyk-Krzeska e Trzebski (Czyzyk-Krzeska e Trzebski, 1990). Eles reportaram que o pico de atividade simpática de ratos espontaneamente hipertensos (SHR) se correlacionava-se com a fase inspiratória do ciclo respiratório (Czyzyk-Krzeska e Trzebski, 1990).

Mais recentemente Simms e colaboradores, proveram uma clara evidência de que ratos SH exibem hiperatividade simpático-respiratória antes do desenvolvimento da hipertensão arterial (Simms *et al.*, 2009). Estes resultados suportam o conceito de uma relação causal direta entre um aumentado acoplamento simpático-respiratório e aumento da resistência vascular periférica em ratos SH.

Em concordância com os experimentos em ratos SH, experimentos com ratos submetidos à hipóxia intermitente crônica (CIH) também tem demonstrado que mudanças no acoplamento simpático-respiratório é o principal mecanismo subjacente ao desenvolvimento de hipertensão arterial nesses animais (Zoccal *et al.*, 2008; Zoccal *et al.*, 2009; Costa-Silva *et al.*, 2012). Prévios estudos têm comprovado que a atividade simpática desses animais apresenta picos de disparos durante as fases Late-I e Post-I (Zoccal *et al.*, 2008), além de um adicional burst de atividade simpática durante a fase late-E, induzindo assim, um aumento na atividade simpática durante a fase expiratória (Zoccal *et al.*, 2008).

Interessantemente essas alterações no padrão de acoplamento simpático respiratório dos ratos CIH foram fortemente associadas com mudanças no padrão respiratório basal, sendo observado um novo e aumentado pico da atividade de nervos abdominais durante a fase late-E (Zoccal *et al.*, 2008). Esses experimentos demonstram que ratos CIH apresentam um padrão de expiração forçada, sugerindo uma relação causal entre ativa expiração e hiperatividade simpática nesse modelo (Zoccal *et al.*, 2008; Costa-Silva *et al.*, 2012; Moraes, Bonagamba, *et al.*, 2014).

Baseado nesses aspectos patofisiológicos, nosso grupo de pesquisa tem avaliado a possibilidade de que alterações nas interações entre neurônios respiratórios e simpáticos podem contribuir para aumentar a atividade simpática basal e predispor o desenvolvimento de hipertensão em ratos submetidos à restrição proteica durante gestação e lactação. Nós inicialmente examinamos essa possibilidade usando ratos não anestesiados e posteriormente a preparação coração-tronco cerebral isolados (CTCI).

Interessantemente, demonstramos que ratos submetidos à restrição proteica durante a gestação e lactação exibem aos 30 dias de vida pressão arterial semelhante aos animais do grupo controle, no entanto aos 90 dias esses animais desenvolveram hipertensão arterial (De Brito Alves, Nogueira, *et al.*, 2014a). Barros e colaboradores, utilizando ratos adultos não anestesiados expostos à restrição proteica perinatal demonstrou que o aumento de pressão arterial aos 90 dias está associado a um aumento no tônus simpático cardíaco. Sugerindo que atividade predominante do sistema nervoso simpático no coração e sistemas vasculares pode contribuir para o aumento de pressão arterial nesses animais (Barros *et al.*, 2015).

Além disso, demonstramos que ratos submetidos à desnutrição proteica durante a gestação e lactação exibem a curto prazo (30 dias) importantes modificações no ritmo respiratório, elucidado por um aumento de frequência respiratória e ventilação pulmonar durante o período basal (De Brito Alves, Nogueira, *et al.*, 2014a). Diante dessas observações, a nossa hipótese é que que possíveis alterações simpático-respiratórias no início da vida podem contribuir para o desenvolvimento de hipertensão arterial em nosso modelo de desnutrição proteica.

Através de procedimentos realizados na preparação CTCI, realizamos o registro direto da atividade elétrica dos nervos simpático e frênico e supreendentemente ratos juvenis provenientes de mães alimentadas com dieta hipoproteica durante a gestação e lactação, exibem, antes do desenvolvimento de hipertensão arterial, aumento na atividade motora simpática e inspiratória basais (De Brito Alves *et al.*, 2015).

Esses achados, possivelmente ajudarão a elucidar os mecanismos pelos quais indivíduos submetidos à desnutrição proteica durante um período crítico da vida apresentam maior predisposição ao desenvolvimento de hipertensão arterial na vida adulta.

A respiração é reflexamente controlada por órgãos especiais sensíveis a mudanças na pressão parcial arterial (Pa) de O_2, de CO_2, ou na concentração de H^+, e que agem para manter esses parâmetros em níveis ideais (Gonzalez *et al.*, 1994; Costa-Silva *et al.*, 2010). Os órgãos sensórios responsáveis por esse controle homeostático são denominados de quimiorreceptores e estão localizados perifericamente, principalmente na bifurcação carotídea e arco aórtico (Biscoe e Purves, 1967; Gonzalez *et al.*, 1994).

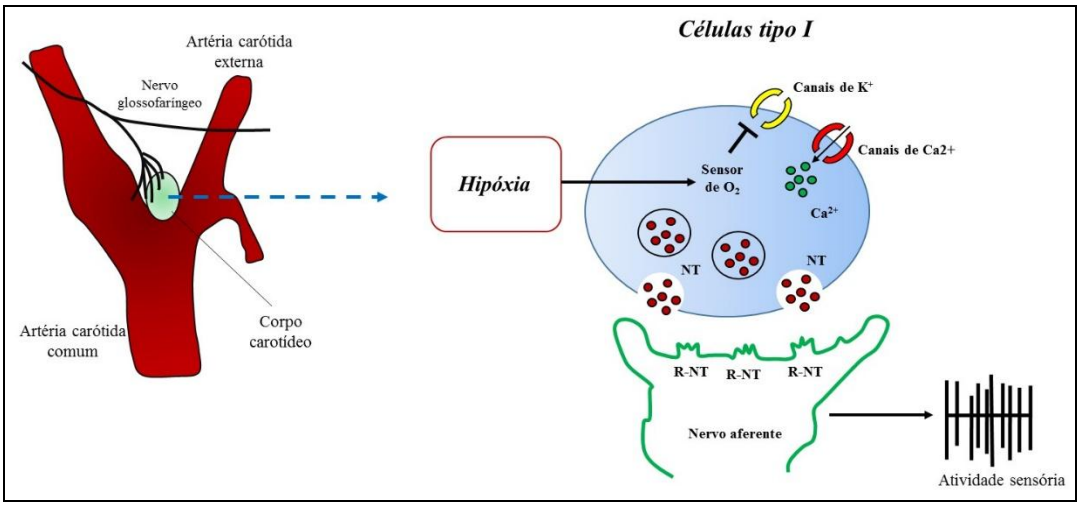
Em 1900 Kohn descreveu a organização do parênquima em ilhotas de células do corpo carotídeo e as denominou de células clusters, células glomus ou corpo carotídeo, principal região onde estão localizados os quimiorrceptores periféricos (Biscoe e Purves, 1967; Gonzalez *et al.*, 1994). Em ratos, o corpo carotídeo (CB) está localizado bilateralmente na bifurcação das artérias carótida comum, recebendo inervação sensória de um ramo do nervo

glossofaríngeo, também chamado de seio do nervo carotídeo (Gonzalez *et al.*, 1994). O CB é formado por 2 tipos de células: células tipo I, principal, células glomus ou quimiorreceptores, estas estão em maior quantidade, apresentam grande número de mitocôndrias (12 a 15% do volume), são positivas para tirosina hidroxilase (TH) e estão em contato com várias terminações nervosas; enquanto as células tipo II ou sustentaculares são positivas para proteína ácida fibrilar glial (GFAP) (De Caro *et al.*, 2013).

O CB não é maduro ao nascer e a sensibilidade ao O_2 não está completamente funcional (Wong-Riley *et al.*, 2013). A resposta das células glomus à hipóxia aumenta com a idade, tornando mais responsiva do dia 1 ao dia 8 de vida (Sterni *et al.*, 1999; Wong-Riley *et al.*, 2013). Após o dia 8º dia de vida, as respostas do corpúsculo carotídeo à hipóxia tornam-se relativamente estável. No entanto, a completa maturidade do corpúsculo carotídeo em ratos só acontece no final da 3 semana de vida pós-natal (Ling *et al.*, 1997; Liu *et al.*, 2009; Wong-Riley *et al.*, 2013). Desde o nascimento até a maturação total do corpúsculo carotídeo ocorre inúmeras mudanças simultâneas e radicais a nível neuroquímico, metabólico, ventilatório e eletrofisiológico (De Caro *et al.*, 2013). Por exemplo, durante a 2º e 3º semana pós-natal há um aumento na expressão de receptores glutamatérgicos, no entanto a expressão de receptores gabaérgicos diminui (Liu e Wong-Riley, 2004; Wong-Riley *et al.*, 2013). Nesse mesmo período a atividade da enzima citocromo oxidase aumenta no corpúsculo e em vários núcleos do tronco cerebral (Liu e Wong-Riley, 2003). Todas essas modificações são importantes para a maturidade do corpúsculo carotídeo e consequentemente capacidade adequada em responder à hipóxia.

Quedas na pO_2 e pH e/ou elevação na pCO_2 (quadros de hipóxia) são detectadas pelos quimiorreceptores (células glômus) e transformados em sinais elétricos. Estes, são enviados, via nervo glossofaríngeo e vago, ao sistema nervoso central, sobretudo em nível bulbar, realizando sinapses de primeira ordem no núcleo do trato solitário comissural (NTS), medial, núcleo motor dorsal do vago (DMNX) e na área postrema (Finley e Katz, 1992) . As frequências de descargas do nervo do seio carotídeo são baixas durante normóxia (PO_2 arterial ~100 mmHg), mas aumentam dramaticamente durante uma modesta queda de PO_2 arterial (~60-80 mmHg) (Prabhakar, 2013).

O exato mecanismo de quimiotransdução induzidos pela hipóxia não estão completamente elucidados, mas ao que parece, ocorre devido a um aumento nas concentrações de cálcio intracelular e liberação de neurotransmissores dependentes de Ca^{+2} das células glômus bem como através da inibição de canais específicos de O_2 sensíveis a K^+ (canais TASK e BK_{Ca}) (Prabhakar, 2013) (**Figura 2**).



Adaptado de Nanduri Prabhakar 2013.

Figura 2. Ilustração esquemática da transdução do estímulo hipóxico nas células tipo I da bifurcação carotídea. **Abreviações**: NT: neurotransmissor, R-NT: receptor do neurotransmissor.

Além disso, muitos neurotransmissores e neuromodulares, a exemplo do ATP, acetilcolina (ACh), ácido gama-aminobutírico (GABA), serotonina (5-hidroxitripitamina), dopamina, noradrenalina, adrenalina, adenosina, encefalinas, neuropeptídeo Y, galanina, endotelina, bombesina, adrenomedulina e leptina tem sido identificado nos grânulos citoplasmáticos das células tipo I e desempenham relevante função na compreensão dos mecanismos de quimiotransdução (Katz *et al.*, 1993; Nurse, 2010; Prabhakar, 2013). Três mensageiros gasosos endógenos também estão envolvidos nos mecanismos de quimiotransdução. Óxido nítrico (NO) liberado pelas fibras eferentes glossofaríngea atua sobre os receptores P2X causando hiperpolarização e consequentemente inibição das células glômus. O monóxido de carbono (CO) inibe a atividade do corpo carotídeo enquanto que o sulfeto de hidrogênio (H_2S) causa ativação dos quimiorreceptores periféricos (Prabhakar, 2013).

Os quimiorreceptores periféricos são ativados principalmente por hipóxia hipóxica (mistura gasosa com 7% de O_2) ou por íons CN^- (hipóxia citotóxica). A ativação dos quimiorreceptores periféricos promove reflexamente hiperventilação, hiperatividade simpática, bradicardia e aumento da pressão arterial, desempenhando assim papel fundamental no controle ventilatório, bem como na homeostase pressórica.

Recentemente, a função dos quimiorreceptores do corpo carotídeo na patofisiologia de doenças cardiovasculares tem ganho considerável interesse (Braga *et al.*, 2006; Abdala *et al.*,

2012; Costa-Silva *et al.*, 2012). Estudos prévios tem demostrado que a simpatoexcitação durante a apneia do sono ou na insuficiência cardíaca pode ser originada de uma maior sensibilidade de quimiorreceptores do corpo carotídeo (Garcia-Rio *et al.*, 2009). Além disso, em ambos pacientes hipertensos e modelos animais de hipertensão, a resposta simpato-excitatória evocada durante ativação do quimiorreflexo periférico está aumentada (Abdala *et al.*, 2012; Sinski *et al.*, 2014).

Em ratos espontaneamente hipertensos (SH) foi demonstrado um aumento na sensibilidade quimiorreceptora do corpo carotídeo quando comparado aos ratos Wistar normotensos (Abdala *et al.*, 2012). Essas alterações podem contribuir para um aumento do tônus simpático vasomotor e consequentemente desenvolvimento da hipertensão arterial (Abdala *et al.*, 2012). Ao que parece essa atividade tônica do CB em ratos SH pode resultar de um aumento na expressão dos canais sensíveis a ácido não dependentes de voltagem - ASIC3 e TASK1, ou também o balanço entre CO_2 e H_2S e a atividade do fator induzível à hipóxia (HIF1α) pode desempenhar uma importante função (Tan *et al.*, 2010; Prabhakar e Semenza, 2012; Prabhakar, 2013). Juntamente a esses achados, modelos animais de insuficiência cardíaca crônica e exposto à hipóxia intermitente tem demonstrado que a atividade dos quimiorreceptores do CB também está aumentada (Marcus *et al.*, 2014).

Recentemente, foi demonstrado que a desenervação do seio carotídeo diminui a pressão arterial em aproximadamente 17 mmHg em ratos espontaneamente hipertensos (Mcbryde *et al.*, 2013), devido possivelmente a uma redução nas descargas do nervo simpático, incluindo o nervo simpático renal, melhoramento do ganho barorreflexo cardíaco, mudanças na excreção renal e redução da infiltração vascular de células T (Mcbryde *et al.*, 2013). Esses dados são comparáveis aos estudos com pacientes hipertensos, no qual a inativação do CB com 100% de oxigênio (hiperóxia) induziu redução na pressão arterial e atividade simpática, suportando assim uma relação causal entre os quimiorreceptores periféricos e o desenvolvimento de hipertensão arterial (Sinski *et al.*, 2014).

Diante das evidências demonstrando o importante papel dos quimiorreceptores periféricos no desenvolvimento de hipertensão, nosso grupo tem testado a hipótese de que uma maior sensibilidade de quimiorreceptores periféricos podem também contribuir para o desenvolvimento de hipertensão arterial em ratos expostos à restrição proteica durante a gestação e lactação.

Recentemente, dados do nosso laboratório demonstraram que ratos malnutridos exibem, aos 30 dias de vida, maior sensibilidade ventilatória ao O_2. Além disso, experimentos realizados em ratos não anestesiados e na preparação coração tronco cerebral isolados

demonstraram maior sensibilidade simpático-respiratória durante a ativação dos quimiorreceptores periféricos (De Brito Alves *et al.*, 2015). Demonstrando que os quimiorreceptores periféricos podem desempenhar também uma importante função no desenvolvimento de hipertensão em ratos expostos à restrição proteica perinatal.

O L-Glutamato (GLU) é o principal neurotransmissor excitatório do sistema nervoso central (Talman *et al.*, 1980; Marmiroli e Cavaletti, 2012). Sinapses glutamatérgicas servem como estações de transmissão excitatórias entre terminais nervosos pré-sinápticos e espinhas dendríticas pós-sináptica (sinapses axo-dendríticas) ou terminações nervosas adjacentes (sinapses axo-axonal) (Niciu *et al.*, 2012). Receptores de glutamato podem ser divididos em duas categorizações: receptores ionotrópicos e metabotrópicos (Marmiroli e Cavaletti, 2012; Niciu *et al.*, 2012).

Receptores glutamatérgicos ionotrópicos formam complexos tetraédricos que são ativados por fluxo de íons (Na^{+} Ca^{+2}). Três classes de receptores ionotrópicos tem sido identificadas: α-amino-3 hodroxi-5-metil-4-isoxazolepropionate (AMPA), N-metil-D-aspartate (NMDA) e cainato (KA) (Niciu *et al.*, 2012). Já os receptores metabotrópicos, são acoplados a proteínas GTP ligadas (proteínas G) e modulam a produção de mensageiros intracelulares (Niciu *et al.*, 2012).

Como descrito anteriormente, a região rostral ventrolateral do bulbo (RVLM) é o principal sitio responsável pela geração da atividade simpática (Guyenet *et al.*, 1990; Dampney, 1994). A atividade de neurônios na RVLM é influenciada por mecanismos excitatórios e inibitórios e um dos principais neurotransmissores excitatórios na RVLM é o L-glutamato (Dampney, 1994) . Portanto o L-glutamato, bem como seus receptores ionotrópicos apresentam importante função na regulação da atividade simpática e cardiovascular (Willette *et al.*, 1983; Guyenet *et al.*, 1989; Bergamaschi *et al.*, 1995; Goodchild e Moon, 2009)

Mapeando a medula oblonga, foi evidenciado 5 regiões importante no controle cardiovascular e respiratório de ratos anestesiados: i) microinjeções de L-glutamato na RVLM/região Bötzinger induziu aumento na atividade do nervo simpático esplânico (ANS), aumento na pressão arterial, além de aumento na frequência e diminuição de amplitude do nervo frênico (NF); ii) L-glutamato na RVLM/região pré-Bötzinger levou um discreto aumento na ANS e pressão arterial, além de diminuição na amplitude do NF; iii) L-glutamato em um sítio medial da RVLM induziu aumento na ANS, na pressão arterial e aumento na frequência e amplitude do NF; iv) microinjeções de L-glutamato na região caudal vetrolateral da medula (CVLM) induziu diminuição na ANS, na pressão arterial, além de diminuição na frequência e amplitude do NF; v) por fim, interessantemente foi demonstrado a presença da

área pressora caudal (APC) e área pressora medulo-cervical (APMC), nas quais microinjeções de L-glutamato induz aumento na ANS, aumento de pressão arterial e na amplitude no NF (Goodchild e Moon, 2009).

Portanto, microinjeções de glutamato na RVLM são capazes de excitar neurônios pré-motores simpáticos levando a um aumento na atividade nervosa simpática e da pressão arterial (Willette *et al.*, 1983; Tolentino-Silva *et al.*, 1997; Moraes *et al.*, 2011). Em contrapartida, o bloqueio de receptores glutamatérgicos na RVLM, elimina muitos reflexos simpato-excitatórios e causa uma diminuição da pressão arterial (Guyenet, 2006; Bardgett *et al.*, 2010).

Além disso, evidências experimentais têm demonstrado que o L-glutamato na RVLM é importante para o controle de parâmetros respiratórios. Por exemplo, tem sido demonstrado que microinjeções de L-glutamato na RVLM de ratos anestesiados induz aumento de frequência respiratória (Monnier *et al.*, 2003). Recentemente, Moraes e colaboradores investigaram a participação da neurotransmissão glutamatérgica em diferentes sub-regiões da RVLM (RVLM/BötC and RVLM/pre-BötC), demonstrando que em ratos não anestesiados microinjeções de L-glutamato no BötC e pre-BötC produz aumentos semelhante na pressão arterial, no entanto L-glutamato no BötC diminui da frequência respiratória, o volume corrente e a ventilação pulmonar, enquanto que L-glutamato no pre-BötC causou aumento na frequência respiratória e ventilação pulmonar. (Moraes *et al.*, 2011).

A potenciação da neurotransmissão glutamatérgica tem sido verificada em vários modelos de hipertensão arterial. Por exemplo, ratos SH tem demonstrado uma maior queda na pressão arterial após microinjeções bilaterais de ácido kinurênico (antagonista não-seletivo de receptores ionotrópicos) na RVLM quando comparados a ratos Wistar-Kyoto (Ito *et al.*, 2000). Experimentos envolvendo ratos sensíveis ao sal tem demonstrado que microinjeções de L-glutamato no RVLM produziu maior resposta pressórica quando comparados aos ratos resistentes ao sal (Ito *et al.*, 2001). Além disso, ratos expostos à hipóxia intermitente crônica como também modelos de insuficiência cardíaca crônica tem demonstrado uma maior modulação excitatória glutamatérgica na RVLM desses animais (Braga *et al.*, 2006; Wang *et al.*, 2009).

Diante desses estudos torna-se claro que a neurotransmissão glutamatérgica, sobretudo na RVLM, desempenha uma importante função na instalação da hipertensão arterial. Tendo em vista dados de nosso laboratório que revelam um aumento na frequência respiratória, na atividade simpática e na pressão arterial em ratos submetidos à desnutrição proteica perinatal,

levantamos a hipótese que esses animais apresentam alteração na neurotransmissão glutamatérgica na RVLM.

Por fim, é provável que o aumento da prevalência de hipertensão arterial na vida adulta pode estar associado ao estilo de vida materno. Neste sentido, uma dieta pobre em proteínas durante a gestação e lactação podem resultar em uma maior predisposição ao desenvolvimento de hipertensão arterial sistêmica na vida adulta dos filhos. Ao que parece, uma maior atividade simpático-respiratória associada a uma maior sensibilidade de quimiorreceptores periféricos são mecanismos importantes na compreensão da origem da hipertensão arterial em indivíduos submetidos à desnutrição durante períodos críticos da vida (**Figura 3**). Contudo, é importante ressaltar que estes efeitos não assumem um caráter determinista, e a prática de hábitos saudáveis pode ter um papel relevante na prevenção do desenvolvimento da hipertensão arterial.

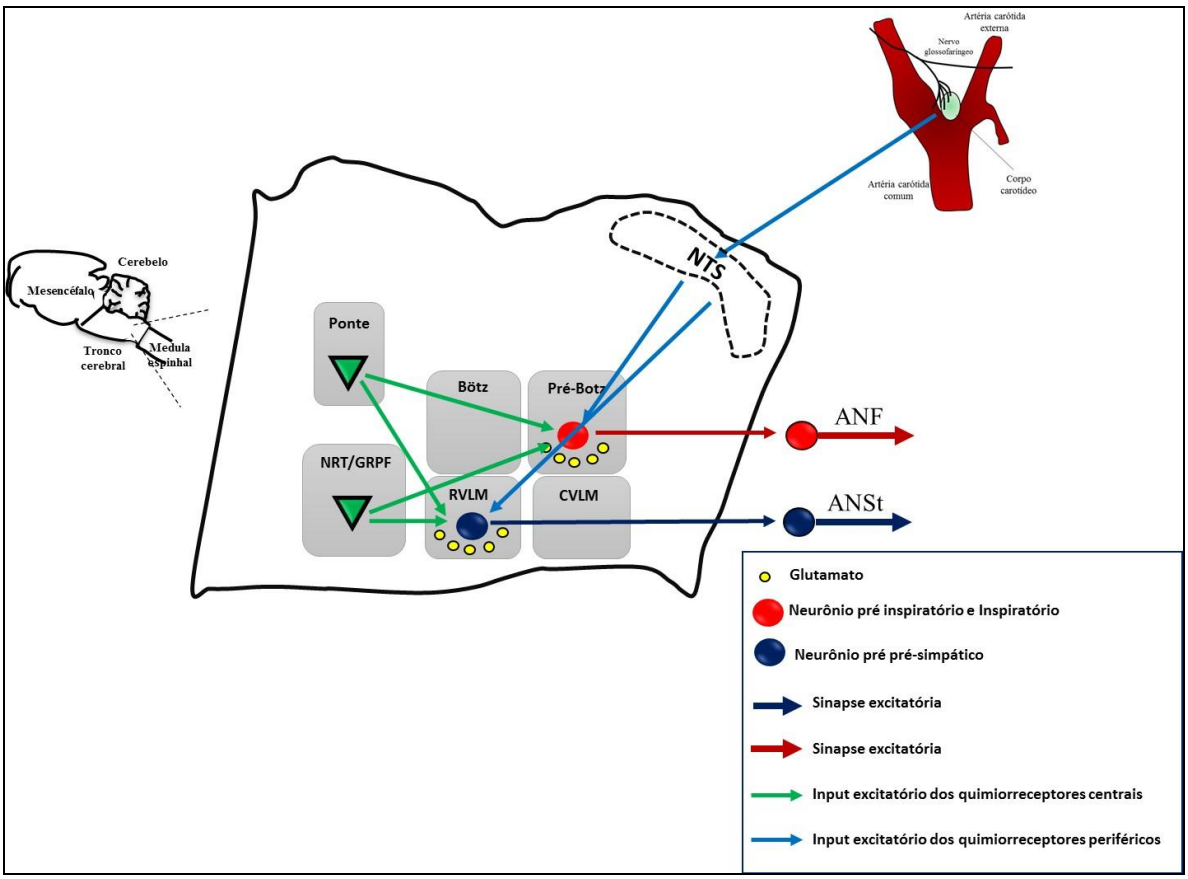


Figura 3. Esquemático modelo de um corte sagital do tronco encefálico de rato mostrando as possíveis alterações em ratos submetidos à desnutrição proteica durante a gestação e lactação que podem contribuir para o desenvolvimento de hipertensão arterial. Os compartimentos do tronco encefálicos apresentados na figura são: Núcleo do Trato Solitário (NTS), complexo Pré-Bötzinger (Pré-Bötz), complexo Bötzinger (Bötz), Núcleo Retrotrapezóide e Grupo Respiratório Parafacial (NRT/GRPF), Região Rostral ventrolateral da medula (RVLM),

Região Caudal Ventro-Lateral da Medula (CVLM); perifericamente está representado a bifurcação carotídea, local onde estão localizados os quimiorreceptores periféricos.

A geração da atividade inspiratória ou atividade do nervo frênico (ANF) ocorre devido sinapses excitatórias, possivelmente glutamatérgica (pequenos círculos em amarelo), enquanto que a atividade simpática ocorre através de sinapses excitatória em neurônicos pré-simpáticos na RVLM, possivelmente também glutamatérgica). A atividade simpática e respiratória é modulada por drives tônicos de quimiorreceptores centrais (triângulo em verde), bem como por aferências excitatórias dos quimiorreceptores periféricos. Ao que parece, ratos malnutridos apresentam hiperatividade simpato-respiratório aos 30 dias. Temos teorizado que isso ocorre devido um aumento intrínseco na atividade de neurônios pré-simpáticos e inspiratórios via neurotransmissão glutamatérgica, associado a inputs excitatórios de quimiorreceptores centrais e periféricos

Ambiente perinatal, expressão gênica e o risco de desenvolvimento de resistência à insulina

Inúmeros achados epidemiológicos e experimentais demonstraram que a restrição proteica durante a gestação e/ou lactação é um importante fator de risco para o desenvolvimento de resistência à insulina e *diabetes mellitus* tipo 2 (DM2) na vida adulta dos filhos (Ozanne *et al.*, 1996; Fernandez-Twinn *et al.*, 2006; Muhlhausler *et al.*, 2009; Reusens *et al.*, 2011; Ashcroft e Rorsman, 2012; Leandro, Fidalgo, *et al.*, 2012).

Durante o estado pós-prandial, a secreção de insulina desempenha uma importante função no controle da homeostase de nutrientes, através de processos anabólicos em vários tecidos (Guo, 2014; Hojlund, 2014). Em geral, a insulina estimula o influxo de glicose dentro do músculo e tecido adiposo, sintetiza proteína e glicogênio no músculo e fígado ou sintetiza e armazena lipídeos no fígado e tecido adiposo. A insulina é responsável pela inibição da oxidação de ácidos graxos, da glicogenólise e gliconeogênese (Randle *et al.*, 1963; Guo, 2014). Já durante o jejum, os níveis de insulina diminuem e hormônios contra-reguladores, a exemplo do glucagon, favorecendo a oxidação de ácidos graxos (lipólise), glicogenólise e gliconeogênse, com a principal finalidade de manter a homeostase glicêmica (Randle *et al.*, 1963; Guo, 2014; Hojlund, 2014).

Prejuízos na função β-pancreática ou na ação da insulina em diferentes órgãos, como músculo, fígado ou tecido adiposo estão intrinsicamente relacionados ao desenvolvimento da resistência à insulina e consequentemente a um quadro de hiperglicemia crônica, a qual define a patogênese do diabetes mellitus tipo 2 (DM2) (Guo, 2014). A hiperinsulinemia é considerada a principal característica na síndrome metabólica, resultado de uma hipersecreção de insulina pelas células β pancreática, sendo reconhecida como um importante fator de risco para o desenvolvimento de DM2 e disfunções cardiovasculares (Jorge *et al.*, 2011; Guo, 2014).

A glicose é o principal substrato energéticos do músculo esquelético (cerca de 75%) em condições normoglicêmicas-hiperinsulinêmica (Randle *et al.*, 1963; Petersen *et al.*, 2003). No entanto, em condições de jejum, o músculo utiliza os ácidos graxos como principal combustível energético

(Randle *et al.*, 1963; Murphy *et al.*, 2004). A relação entre o metabolismo de glicose e ácido graxo no músculo é conhecida com ciclo de Randle e tem sido demonstrado que alterações na homeostase glicose-ácido graxo muscular pode contribuir para o desenvolvimento de resistência à insulina e predispor o DM2 (Randle *et al.*, 1963; Rodgers, 1998; Brownsey *et al.*, 2006; Da Silva Aragao *et al.*, 2014).

Tem sido descrito que um aumento nas concentrações de glicose induz aumento na secreção de insulina, a qual suprime a liberação de ácidos-graxos não esterificado do tecido adiposo (Randle *et al.*, 1963). Isso reduz a competição de ácidos graxo para utilização como substrato e a glicose torna-se o principal combustível (Randle *et al.*, 1963). Em condições de jejum, quando as concentrações de glicose e insulina estão baixas, ocorre um aumento nas concentrações de ácidos-graxos não esterificados, os quais são utilizados como principal substrato energético pelo músculo esquelético. Portanto, quando se aumenta oxidação de ácidos-graxos no músculo menos glicose será então utilizada, enquanto que altos níveis de glicose e insulina suprimem a oxidação de ácidos-graxos (Randle *et al.*, 1963).

Previamente, tem sido demonstrado que vários metabólitos derivados da oxidação de ácido-graxos inibem a atividade de enzimas glicolíticas (Randle *et al.*, 1963). Por exemplo, a elevação de acetil-CoA suprime a atividade do complexo piruvato desidrogenase (PDH); um aumento nos níveis de citrato inibe a fosfofrutoquinase (PFK); e o acúmulo de glicose-6-fosfato (G6P) em geral inibe a atividade da hexoquinase 2 (HK2). Nessa perspectiva, tem sido evidenciado que a oxidação de lipídios no musculo esquelético é coordenado pelo aumento na expressão gênica da lipoproteína lipase 1 (LPL1), carnitina palmitoil transferase 1 (CPT1), proteína desacopladora 3 (UCP3) associado também com maior expressão de enzimas da via da beta oxidação, a exemplo da beta-hidroxi-acilCoA-desidrogenase (βHAD).

O fígado desempenha uma função chave no metabolismo energético do corpo, atuando metabolicamente conectado a vários tecidos, a exemplo do músculo esquelético e tecido adiposo (Matsuda, 1966; Kwong *et al.*, 2007; Rui, 2014). Em uma situação de ingestão alimentar, produtos glicolíticos são utilizados na síntese de ácidos graxo (lipogênese) (Rui, 2014). Já durante curtos períodos de jejum, o fígado produz e libera glicose através da quebra do glicogênio (glicogenólise) ou via gliconeogênese usando lactato, piruvato, glicerol e aminoácidos (Matsuda, 1966; Rui, 2014).

A enzima fosfoenol-piruvato carboxilase quinase C (PEPCK-C) desempenha uma importante função no processo de gliconeogênese, convertendo o oxalacetato citoplasmático em fosfoenol-piruvato, o qual após múltiplas reações bioquímicas é convertido em frutose 1,6-bifosfato e posteriormente frutose-6-fosfato. Uma outra importante enzima no processo da gliconeogênese é a glicose-6-fosfatase (G6P), enzima responsável pela conversão da glicose-6-fosfato em glicose (Rui, 2014). Prévios trabalhos apontam que uma desregulação no processo de gliconeogênese hepática está associada com maior predisposição ao desenvolvimento de resistência à insulina e DM2 (Jia *et al.*, 2012; Ramadan *et al.*, 2013)

Altas concentrações de glicose no fígado induz a expressão de transportadores glicolíticos e de enzimas lipogênicas, a exemplo da enzima sintase de ácidos graxos (FAS), o qual é respondável pela síntese *de novo* ácidos-graxos (Meugnier *et al.*, 2007). Estudos clínicos e experimentais tem demonstrado que aumento na lipogênese hepática está estreitamente associado com maior predisposição de desenvolvimento de resistência à insulina e DM2 (Kumashiro *et al.*, 2011; Jensen-Urstad e Semenkovich, 2012).

Resumidamente, esses achados demonstram que alterações metabólicas no músculo esquelético, fígado e tecido adiposo são importantes para desenvolvimento de resistência à insulina, DM2 e doenças cardiovasculares. Logo, o entendimento de como ocorre essas disfunções metabólicas, certamente serão relevantes para um melhor entendimento destas patologias.

Prévios trabalhos têm demonstrado que alterações na expressão de genes metabólicos envolvidos no metabolismo de lipídios e glucose no músculo, fígado e tecido adiposo podem contribuir para o desenvolvimento de resistência à insulina e DM2 (Mortensen *et al.*, 2010). Nessa perspectiva, foi observado que ratos expostos à restrição proteica durante a gestação e lactação exibem pronunciado efeito sobre a expressão gênica no músculo e fígado. Particularmente, genes envolvidos com fosforilação oxidativa são pouco expressos no músculo esquelético e com elevada expressão no fígado de ratos expostos à restrição proteica perinatal (Mortensen *et al.*, 2010).

Nessa direção, da Silva Aragão e colaboradores (2014), observaram que ratos adultos submetidos à restrição proteica durante a gestação e lactação apresentaram redução na atividade enzimática da HK2 no músculo soleus quando comparados ao grupo controle. Sugerindo consequentemente, uma menor atividade glicolítica ao nível muscular (Da Silva Aragao *et al.*, 2014). Jia e colaboradores (2012) observaram em porcos que a restrição proteica durante a gestação acarreta um aumento na expressão de G6Pase no fígado da prole, a qual foi acompanhada por um processo de hipometilação na região promotora da G6Pase e por hiperacetilação em histonas H3. Sugerindo que a restrição proteica materna causa uma ativação hepática da expressão de G6Pase na prole, a qual pode contribuir para quadros de hiperglicemia e resistência à insulina (Jia *et al.*, 2012).

Portanto, os achados demonstram que ratos expostos à restrição proteica durante a gestação e lactação apresentam maior predisposição de desenvolver hipertensão arterial e doenças metabólicas na vida adulta. Por isso, o entendimento dos mecanismos subjacentes envolvido em patologias como hipertensão, resistência à insulina, DM2 e obesidade, certamente serão relevantes para o desenvolvimento de futuras medidas terapêuticas que auxiliem na prevenção de doenças e agravos não transmissíveis em indivíduos submetidos à desnutrição no início da vida.

Nessa perspectiva, nosso trabalho procurou evidenciar quais os possíveis mecanismos envolvidos no desenvolvimento de hipertensão em ratos expostos à restrição proteica perinatal, como também, possíveis modificações na expressão gênica e proteica de enzimas envolvidas no metabolismo de glicose e ácido-graxo no músculo esquelético e fígado em ratos jovens e adultos, as quais poderiam auxiliar na compreensão de maior um risco ao desenvolvimento de resistência à

insulina.

3. HIPÓTESE EXPLORADAS NO PRESENTE ESTUDO

I - Diante das divergências existentes na literatura acerca dos mecanismos envolvidos no desenvolvimento de hipertensão arterial em ratos expostos à restrição proteica perinatal, testamos a hipótese de que ratos submetidos à restrição proteica durante a gestação e lactação desenvolvem hipertensão arterial sistêmica decorrente de uma maior atividade simpática e respiratória, a qual em parte, é associada a uma maior excitabilidade de quimiorreceptores periféricos e aumentada neurotransmissão glutamatérgica.

II - Vários estudos têm destacado que ratos expostos à restrição proteica perinatal apresentam maior risco de desenvolvimento de resistência à insulina decorrente de disfunções na expressão de genes e proteínas envolvidos no metabolismo de glicose e ácidos-graxos. Nesse cenário, a nossa hipótese foi que ratos expostos a restrição proteica durante a gestação e lactação exibem, a curto (30 dias) e longo prazo (90 dias), alterações na expressão de genes e proteínas envolvidos no metabolismo de glicose e ácidos-graxos no músculo esquelético e no fígado.

4. OBJETIVOS

Geral

Investigar os mecanismos de controle da atividade simpática e respiratória e o perfil de expressão de genes metabólicos e proteínas envolvidos no metabolismo de ácidos-graxos e glicose no músculo e fígado de ratos expostos à restrição proteica durante a gestação e lactação.

Específicos

Em ratos machos provenientes de mães submetidas à restrição proteica durante gestação e lactação:

a) Avaliar a pressão arterial e frequência respiratória em ratos não anestesiados;
b) Analisar a atividade nervosa simpática e respiratória na preparação coração tronco-cerebral isolados;
c) Investigar a sensibilidade dos quimiorreceptores periféricos e centrais;
d) Investigar a participação do corpúsculo carotídeo na gênese da hipertensão arterial;
e) Avaliar as respostas autonômicas após administração de L-glutamato na RVLM;
f) Averiguar a participação de receptores ionotrópicos glutamatérgicos na RVLM no controle cardiovascular e respiratório;
g) Avaliar a expressão gênica e proteica de enzimas envolvidas no metabolismo de glicose-ácido graxo.

5. Materiais e métodos

1. Animais

Foram utilizados ratos machos Wistar do biotério do Centro Acadêmico de Vitória, procedentes de mães que passaram ou não por restrição proteica durante a gestação e lactação. Os animais foram mantidos em gaiolas de polipropileno (4 animais/gaiola), com água filtrada e ração *ad libitum*. Eles foram mantidos em ciclo claro escuro de 12h e com temperatura (22 a 25°C) e umidade (55 a 65 %) controladas. Todos os protocolos e procedimentos experimentais foram realizados de acordo com o Colégio Brasileiro de Experimentação Animal (COBEA) e aprovados pelo Comitê de Ética em Experimentação Animal (CEEA) do Centro de Ciências Biológicas da UFPE (processo n° 23076.044454/2010-94 e processo nº 23076.019345/2013-61) e também pelo comitê de ética em experimentação animal da escola de odontologia da Universidade do Estado de São Paulo – UNESP/Araraquara (protocol 21/2012).

2. Dietas

Foram elaborados dois tipos de dietas a base de caseína: uma normoprotéica (17% de proteína) e outra hipoproteica (8% de proteína). Ambas as dietas foram produzidas no Departamento de Nutrição da Universidade Federal de Pernambuco de acordo com a AIN – 93 (Reeves *et al.*, 1993). As dietas são isocalóricas com alteração apenas no conteúdo proteico conforme a **tabela 1**.

Tabela 1. Composição nutricional das dietas (g/100g dieta).

Nutriente	Grupo NP (17% proteina)	Grupo HP (8% proteina)
Caseina (85%)*	20	9.41
Amido dextrinizado	13	13.2
Celulose	5	5
Sacarose	10	10
Amido de milho	39.74	50.34
Óleo de soja	7	7
Colina	0.25	0,25
Metionina	0.3	0.3

Mix vitamínico	1	1
Mix minaral	3.5	3.5
Valor energético total (Kcal)	3.88	3.88

* A caseína utilizada na preparação da dieta apresentou 85% de pureza

3. Indução da desnutrição proteica durante o período peri-natal (gestação e lactação)

Ratas Wistar virgens com 90 - 120 dias de vida ou com peso acima de 200g foram acasaladas com ratos machos férteis na proporção de 2:1. A observação da presença de espermatozoides no esfregaço vaginal foi utilizada para definir o 1° dia de prenhez.

Posteriormente, as ratas foram colocadas em gaiolas individuais e alocadas randomicamente em dois grupos: grupo normoprotéico (NP, recebeu dieta com 17% de proteína) e grupo hipoprotéico (HP, recebeu dieta com 8% de proteína) durante a gestação (21 dias) e lactação (21 dias). Ao 2º dia de vida, as proles provenientes destas fêmeas foram reduzidas a oito ratos machos por ninhada. Ao 22º dia de vida, todos os filhotes receberam dieta normoprotéica (Labina, Purina Agribands). Após o desmame as ratas fêmeas e os machos utilizados para obtenção da prole foram eutanasiados com overdose de pentobarbital sódico (70 mg/Kg ip). Além disso, a prole de fêmeas também foram eutanasiadas com overdose de pentobarbital sódico (70 mg/kg ip). Nos casos nos quais as ninhadas apresentaram menos de oito ratos machos, ratas fêmeas foram utilizadas para padronização do tamanho da ninhada. Os estudos funcionais foram realizados ao 30° ou 90° dia de vida.

4. Procedimentos para avaliação da ventilação pulmonar, pressão arterial e frequência cardíaca e em animais acordados

As medidas de ventilação foram obtidas por pletismografia de corpo inteiro, em um sistema fechado (Malan, 1973). Durante a realização de cada medida de ventilação, o fluxo de ar é interrompido e a câmara do animal permanece totalmente vedada por curtos períodos de tempo (~2 min). As oscilações causadas pela ventilação do animal foram captadas por um dispositivo conectado à câmara que contém o transdutor diferencial de pressão e o amplificador de sinais (ML141 spirometer, PowerLab, ADInstruments). O sinal foi então enviado para o sistema de aquisição e análise dos dados (LabChartTM Pro, PowerLab, ADInstruments). A calibração do volume foi obtida durante cada experimento, injetando-se

um volume conhecido de ar (1 mL) dentro da câmara do animal com o uso de uma seringa graduada. Em seguida foi registrado as medidas de frequência respiratória (f).

Ao 30° ou 90° dia de vida, os animais foram anestesiados com ketamina (80 mg/kg, i.p.) e xilazina (10 mg/kg, i.p.) para inserção de cateteres de polietileno na artéria e veia femoral, para registro da PA e infusão de drogas, respectivamente. O cateter foi exteriorizado subcutaneamente até a altura do pescoço para facilitar a conexão dele ao transdutor de pressão. Após a cirurgia, os animais receberam uma dose de cetoprofeno (5 mg/kg ip, anti-inflamatório).

O registro da PA e da frequência cardíaca (FC) foi realizado 24 horas após o procedimento cirúrgico em animais não anestesiados por meio da conexão da cânula da arterial femoral com o transdutor mecanoelétrico de pressão, cujo sinal foi devidamente amplificado (ML866/P, ADInstruments, Power Lab, Bella Vista, NSW, Australia), digitalizado por meio de uma interface analógico/digital e amostrado a 2000 Hz em um microcomputador equipado com um software apropriado (LabChartTM Pro, ADInstruments, Bella Vista, NSW, Austrália), para posterior análise. A pressão arterial média (PAM) e FC foram derivadas da pressão arterial pulsátil (PAP) por meio deste sistema de aquisição.

Uma avaliação indireta da modulação autonômica da resistência vascular e da função cardíaca foi realizada através da análise da variabilidade da pressão arterial e da frequência cardíaca no domínio da frequência (Zoccal *et al.*, 2009).

Oscilações de pressão arterial e frequência cardíaca na faixa de baixa frequência (LF) são representativos dos efeitos moduladores da atividade simpática, enquanto oscilações na escala de alta frequência (HF) estão associados a uma modulação respiratória ou parassimpática dos vasos sanguíneos e do coração, respectivamente (Malliani *et al.*, 1991b; Bernardi *et al.*, 2001; Zoccal *et al.*, 2009). No presente estudo, as magnitudes dos efeitos moduladores autonômicos e respiratórios no sistema cardiovascular foram avaliadas em ratos NP e HP.

Inicialmente, foram efetuados registros basais da PA e FC de ambos os grupos durante 1 hora. Os trechos de registros foram divididos em períodos de segmentos de 350 batimentos e então realizada análise espectral auto regressiva, a fim de determinar os componentes oscilatórios de baixa-frequência (LF, 0.20-0.75 Hz) e de alta-frequência (HF, 0.75-3.0 Hz) do registro da pressão arterial e da frequência cardíaca, através de software apropriado (Cardioseries Software v2.4, available on https://www.sites.google.com/site/cardioseries/home).

Para análise no domínio da frequência (análise espectral) da pressão arterial sistólica (PAS) e do intervalo de pulso (IP), uma série de valores basais desses parâmetros foram convertidos em pontos de dados a cada 100 ms usando interpolação cúbica e os dados foram divididos em conjuntos de sequências sobrepostos de 512 pontos de dados (51.2s). Todos os segmentos foram visivelmente inspecionados, e os dados não estacionários foram descartados. O espectro de cada segmento foi calculado usando uma Transformada Rápida de Fourier direta. Os espectros foram integrados em baixa (LF: 0.2-0.75 Hz) e alta frequência (HF: 0.75-3.0 Hz). Flutuações nas bandas de LF e HF além dos valores absolutos (bpm^2) foram também expressos em unidades normalizadas (nu) (Reyes Del Paso *et al.*, 2013). Para avaliação do índice simpato-vagal, a relação LF/HF da variabilidade foi calculada. Oscilações menores do que 0,20 Hz não foram quantificadas.

5. Ativação dos quimiorreceptores periféricos

Hipóxia citotóxica

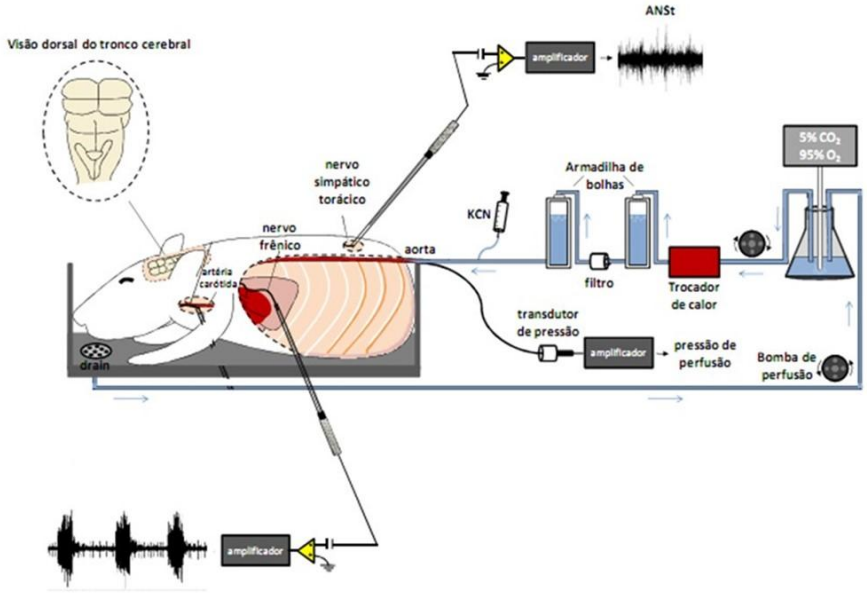
Para estimular os quimiorreceptores periféricos e, consequentemente, ativar o quimiorreflexo foi utilizado o KCN 0,04 %, 100 ul/rato, conforme previamente descrito (Franchini e Krieger, 1993; Machado e Bonagamba, 2005), administrado por via endovenosa, através de um cateter venoso previamente implantado, e as respostas respiratórias e autonômicas foram registradas continuamente.

6. Procedimentos para avaliação da atividade do nervo simpático torácico e frênico na preparação coração tronco cerebral isolados (CTCI)

Os procedimentos realizados na preparação CTCI foram fundamentados na descrição original de Paton (1996) (Paton, 1996). Inicialmente, o animal foi anestesiado pela inalação de halotano (Astra Zeneca, Cotia, SP, Brasil). No momento em que o animal não mais respondia aos estímulos realizados sobre a pata, foi feita a transecção sub-diafragmática e, logo em seguida, o animal foi imerso no fluido cérebro-espinhal artificial [ACSF, composto por (em mM): 125 de NaCl; 24 de $NaHCO_3$; 5 de KCl; 2,5 de $CaCl_2$; 1,25 de $MgSO_4$; 1,25 de KH_2PO_4 e 20 de dextrose] resfriado (~2°C) e aerado com carbogênio (95% de O2 e 5% de CO2). Logo após, o animal foi descerebrado ao nível pré-colicular e a pele foi totalmente removida. Os pulmões foram removidos e o nervo frênico isolado, cortando-o junto ao diafragma. A aorta descendente foi isolada para posterior canulação. A porção lateral superior esquerda da caixa torácica foi removida ao nível torácico médio para melhor exposição do coração, do nervo frênico e do nervo simpático torácico. A exposição da superfície dorsal do

tronco cerebral foi realizada pela remoção do osso occipital, da duramáter e do cerebelo. A preparação foi então transferida para a câmara de registros. A aorta descendente foi canulada e, perfundida retrogradamente com ACSF por uma bomba peristáltica (Watson-Marlow 502S, Falmouth, Cornwall, Inglaterra) a um fluxo de 21-25 mL por minuto. Além disso, foram acrescidos ao ACSF lactato (2 mM), um agente oncótico (Polietilenoglicol, 1,25%, Sigma, St Louis, MO), um bloqueador neuromuscular (vecurônio 3-4 µg/mL, Cristalia, Itapira, SP, Brazil) e a vasopressina (6-12 nM, Sigma, MO, EUA). A vasopressina foi adicionada à solução de perfusão para aumentar a resistência vascular e, consequentemente, ajudar a manter a pressão de perfusão entre 50 - 70 mmHg. O perfusato foi constantemente aerado com carbogênio (95% de O_2 e 5% de CO_2) e aquecido por meio de um trocador de calor a uma temperatura de aproximadamente 32°C.

Afim de prevenir a passagem de coágulos sanguíneos e restos de tecidos em suspensão para preparação, o perfusato foi filtrado por um pré-filtro de polipropileno com poros de 25 µm (Millipore, PP25, Billirica, MA, EUA). Finalmente, o perfusato passava por um sistema que visava evitar a passagem de bolhas de ar para preparação e amortecer as pulsações geradas pela bomba peristáltica. Todos os tubos do sistema eram relativamente impermeáveis ao O_2 e ao CO_2 (Tygon Cole Palmer, Il, EUA, ID: 1,56 mm; OD: 4,7 mm). A porção final do tubo era uma cânula de duplo lúmen (Portex, MA, EUA, ID: 0,28 mm; OD: 0,61 mm), sendo um para a perfusão e outro para o registro da pressão de perfusão (PP). Essa cânula era conectada ao transdutor de pressão (PT 300, Grass Instruments, West Warwick, EUA), que enviava o sinal para o amplificador (15A12, Grass Instruments, West Warwick, EUA) e deste para o computador, onde era realizada a aquisição e o registro dos dados pelo programa Spike 2 (Cambridge Eletronic Design, Cambridge, Inglaterra).



Esquema 1. Representação esquemática da preparação coração-tronco cerebral isolados, adaptada de **Zoccal e cols. (2009) (Zoccal *et al.*, 2009)**, mostrando a localização e os registros das atividades dos nervos frênico (ANF) e simpático torácico (ANSt) registrados, e o sistema de perfusão.

A atividade elétrica de todos os nervos foi obtida usando eletrodos de sucção de vidro apoiados em micromanipuladores (Narishige, Tokyo, Japão). A atividade do nervo frênico esquerdo foi registrada por um eletrodo unipolar e a sua atividade em rampa nos deu um índice contínuo de viabilidade da preparação. Os registros do simpático torácico esquerdo (ao nível T8–T12) foi obtido por eletrodo bipolar. Todos os sinais foram amplificados, filtrados (0.05–5 kHz) e adquiridos por um conversor A/D (CED 1401, Cambridge Electronic Design, Cambridge, UK) para o computador através do software Spike2 (version 7, CED, Cambridge, UK).

6.1 Exposição à hipóxia citotóxica na preparação coração tronco-cerebral isolados

Os quimiorreceptores periféricos foram estimulados pela administração de cianeto de potássio (KCN 0.05 %, 50 µL/rato) através da aorta descendente por meio de uma seringa acoplada ao sistema de perfusão da preparação CTCI, como descrito anteriormente (Braga *et al.*, 2006; Zoccal *et al.*, 2009; Costa-Silva *et al.*, 2010). A estimulação dos quimiorreceptores periféricos pelo KCN 0.05 % produziu respostas ventilatórias e autonômicas consistentes, as quais apresentaram baixa variabilidade entre as preparações.

6.2. Exposição à hipercapnia na preparação coração tronco-cerebral isolados

Para esse protocolo foi utilizado um misturador de gás para que a proporção dos gases no perfusato fosse alterada para aumentar os níveis de CO_2. Para os estímulos hipercápnicos, as concentrações foram de 7% de CO_2 e 93% de O_2 e 10% de CO_2 e 90% de O_2. A duração do tempo de exposição para cada estímulo foi de 5 minutos.

7. Procedimentos para avaliação do papel dos quimiorreceptores periféricos sobre a gênese da hipertensão arterial em animais adultos

Ao 25° dia de vida, animais do grupo normoprotéico e do grupo hipoprotéico foram anestesiados com ketamina (80 mg/kg, i.p.) e xilazina (10 mg/kg, i.p.), e usando técnica cirúrgica com rigorosa assepsia, foi realizada uma incisão na linha média, no nível cervical próximo à traqueia do animal. Os músculos esternóides e esternocleidomastóideo foram cuidadosamente retraídos. Posteriormente, as artérias carótidas foram dissecadas e isoladas, permitindo a visualização da artéria carótida comum e sua bifurcação em carótidas interna e externa. Nesta região, encontra-se o corpúsculo carotídeo. O nervo do seio carotídeo e suas ramificações foram seccionados e as estruturas adjacentes como nervo vago, nervo glossofaríngeo, nervo laríngeo, cadeia simpática cervical e o gânglio simpático cervical superior foram preservadas; em seguida, a incisão foi suturada. Os animais submetidos a desenervação do corpúsculo foram chamados de **cbd** ou "carotid body denervation", enquanto que animais do grupo **sham** passaram pelo mesmo procedimento cirúrgico, mas o corpúsculo carotídeo permaneceu intacto. Após os procedimentos cirúrgicos, os animais de ambos os grupos receberam injeção de penicilina G (50,000 IU sc) e cetoprofeno (5 mg/kg ip).

Não ocorreu mortalidade após o procedimento cirúrgico e os animais ganharam peso normalmente. Para avaliar a integridade da desenervação do corpúsculo carotídeo, respostas ventilatórias a hipóxia sistêmica (7% de O2 de mistura gasosa por aproximadamente 3 minutos) foi realizada por pletismografia de corpo inteiro 10 dias após a remoção do corpo carotídeo, bem como pela ausência de respostas pressóricas e de frequência cardíaca durante administração endovenosa de cianeto de potássio (KCN; 40 μg/0,1 mL/ rato) no dia dos experimentos (Abdala *et al.*, 2012).

Ao 89° dia de vida, foi realizado o procedimento para implante dos cateteres de polietileno na artéria e veia femoral, para registro da PA e infusão de KCN (0,04%, 100uL/rato) e hexametônio (bloqueador pré-ganglionar simpático, 25 mg/Kg), respectivamente.

Vinte e quatro horas após, foi realizado o registro da pressão arterial, da frequência cardíaca e da frequência respiratória durante um período basal de 1 hora e após administração cianeto de potássio (KCN 0.04%) e hexametônio (25 mg/Kg).

A análise de variabilidade da pressão arterial sistólica e da frequência cardíaca também foram avaliadas nesse experimento.

8. Ensaios moleculares

Coleta dos tecidos

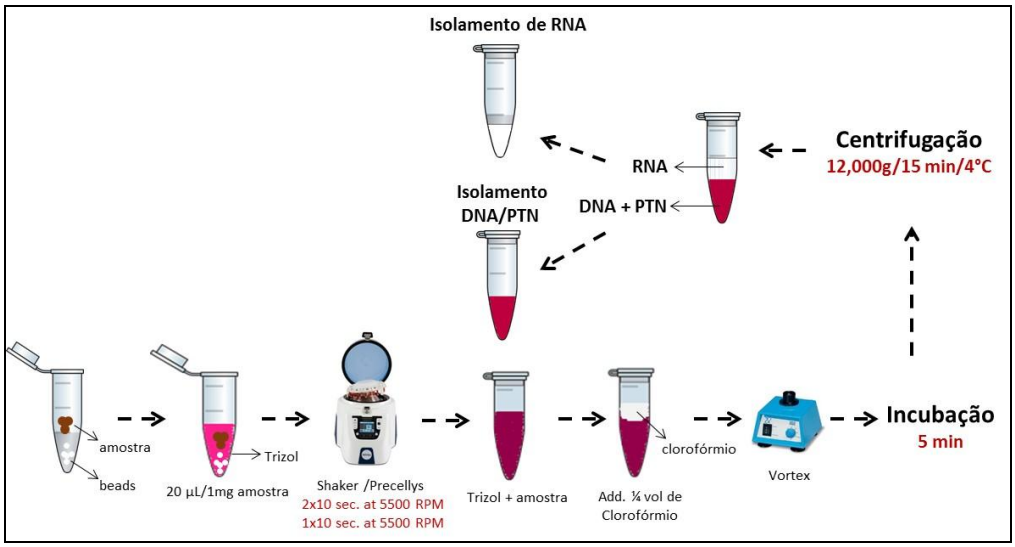
Aos 30 e 90 dias de vida, ratos que não passaram por procedimentos cirúrgicos foram eutanasiados por decapitação para coleta do tronco encefálico, músculo sóleo (oxidativo), músculo extensor longo dos dedos – EDL (glicolítico) e fígado para realização dos ensaios moleculares.

Os presentes tecidos foram coletados, congelados em nitrogênio líquido e imediatamente armazenados em freezer -80°C até a realização das análises.

Extração Tríplice: DNA, RNA e proteínas para análises moleculares

Esse protocolo foi padronizado durante realização do estágio doutoral no instituto CarMeN – Lyon – França, sob direção do Dr. Luciano Pirola (de Brito Alves, JL e Pirola, L).

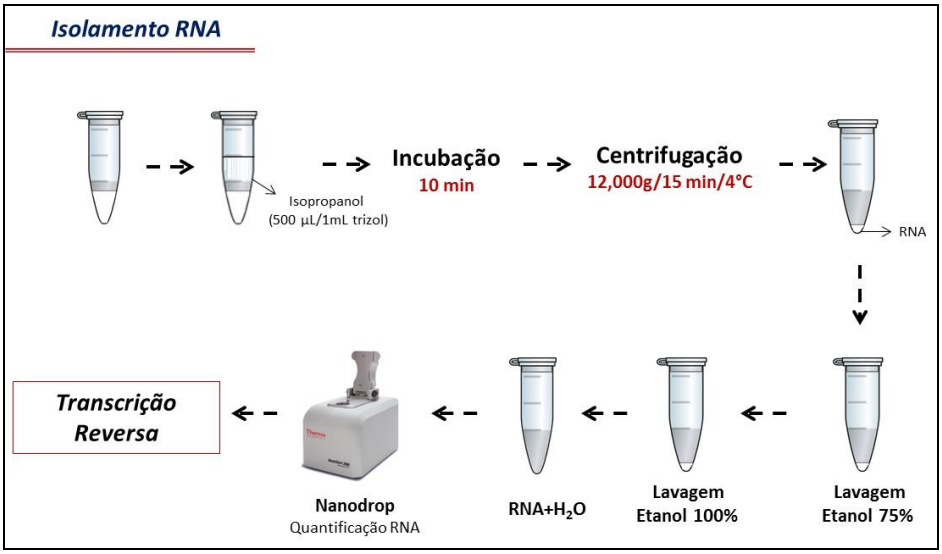
Em uma única amostra de tecido foi adicionado Trizol (Tripure Isolamento Reagente, Roche) para extração de DNA, RNA e proteína. A solução resultante foi transferida para tubos de rolamento (Bertino, Precellys Lise Kit) e homogeneizadas (Bertino, Precellys 24). Em seguida, foi adicionado ¼ do volume de trizol de clorofórmio e realizado centrifugação durante 15 minutos a 13000 RPM. Após a centrifugação, a fase aquosa contendo o RNA foi recolhida e precipitada em isopropanol (0.5mL para cada 1mL de trizol) e a fase contendo trizol (DNA e proteínas) foram utilizadas para extração desses componentes. O esquema 2 ilustra o protocolo realizado para extração tríplice.



De Brito Alves, JL

Esquema 2. Representação esquemática da extração tríplice de RNA, DNA e proteínas.

Após centrifugação do RNA com o isopropanol, o sedimento foi lavado duas vezes com 1 volume de etanol a 75% e 100%. O sedimento de RNA foi ressuspenso em 100 uL de H_2O (Versol). A concentração de RNA foi medida num espectrofotômetro Nanodrop2000, conforme apresentado abaixo no esquema 3.

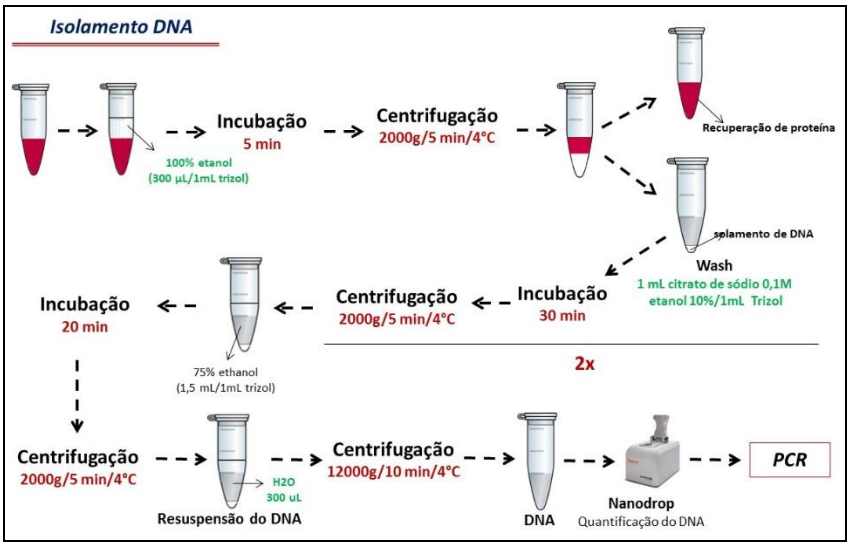


De Brito Alves, JL

Esquema 3. Representação esquemática da extração e leitura do RNA.

O DNA foi isolado na interfase fenol-clorofórmio. Para isso, foi adicionado 0.3 mL de etanol 100% para cada 1mL de Trizol usado no início da reação e centrifugado durante 5 minutos a 7000 RPM. O sobrenadante foi coletado em um novo tubo para extração de proteínas e o pellete precipitado foi lavado em 0.1M de citrato de sódio em etanol 10%. Por

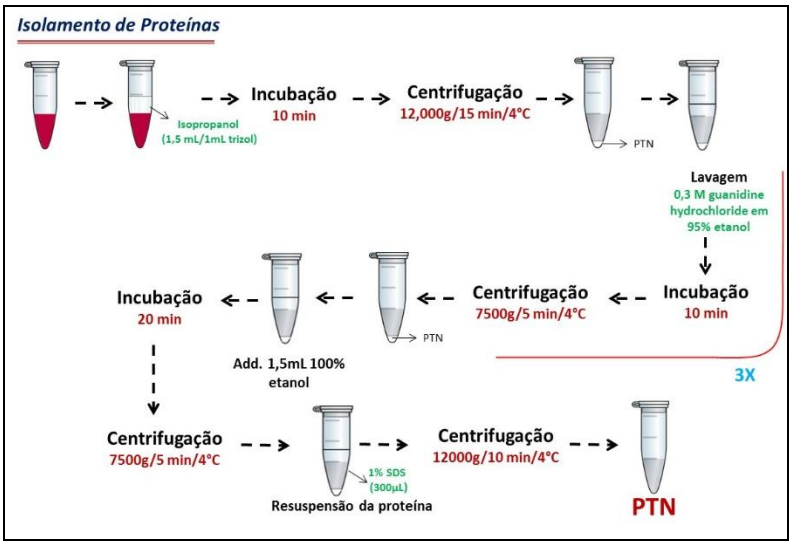
fim, os pelletes foram lavados com etanol 75% e ressuspenso em água. A leitura da quantidade de DNA foi realizada em nanodrop, conforme o esquema 4.



De Brito Alves, JL

Esquema 4. Representação esquemática da extração e leitura do DNA

A extração de proteínas foi realizada no sobrenadante fenol-etanol. Para isso, foi adicionado 1.5mL de isopropanol para cada 1mL de trizol utilizado no início da reação e em seguida centrifugação durante 10 minutos a 13000 RPM. Após, os pelletes foram lavados com guanidina hidrocloride (0.3M) em etanol 95%. Ao final, os pelletes também foram lavados com etanol 100% e as proteínas solubilizadas em SDS 1%, conforme o esquema 5.



De Brito Alves, J.L.

Esquema 5. Representação esquemática da extração de proteínas.

Transcrição reversa RT-TAKARA

A transcrição reversa RT-TAKARA para amostra de RNA foi preparado com 1µg de RNA. A amostra foi então aquecida durante 10 minutos a 65°C e foram adicionados 4µL PrimeScript tampão 5x, 1 ul de mistura de enzimas PrimeScript RT, 1 ul de oligodT (50 microns), 4µl hexâmeros aleatórios (100 uM). RT-ciclo compreende TAKARA 15 minutos a 37°C e 15 segundos a 85°C. A amostra foi então colocada durante 1 minuto em gelo e, em seguida, passou por centrifugação rápida a 4°C. Em seguida, foram adicionados RNase H (1ul) para cada 20 uL de RT puro. Após, RT puro + RNAH foram colocados para incubar durante 20 minutos a 37°C. RT foi diluído a 1/10 por adição de 179µL de H_2O e os tubos armazenados a -20° C. Em seguida, RT 1/10 foi diluído para RT 1/60 (10 ul RT 1/10 + 50 uL H_2O) e a técnica de reação em cadeia da polimerase (PCR em tempo real) foram realizadas para investigação da expressão gênica.

A sequência dos primers utilizados para realização dos experimentos de RT-PCR do presente estudo encontram-se descritas na **tabela 2**.

Tabela 2. Sequência de primers utilizados para realização dos RT-PCR

Gene	Foward/Reverse	Tm	Sequência 5'-3'	Amplicon tamanho, bp
Catalase	F	60°C	CATCGGCACATGAATGGCTA	281 pb
	R		ACCTTGGTCAGGTCAAATGG	
SOD 1	F	58°C	TGAAGAGAGGCATGTTGGAG	164 pb
	R		CCACCTTTGCCCAAGTCATC	
SOD 2	F	60°C	TCATGCAGCTGCACCACAGC	138 pb
	R		CCATTGAACTTCAGTGCAGG	
Grin 1	F	60°C	TACAACTGGAACCACATCATCC	145 pb
	R		TGTCATAGGACAGTTGGTCGAG	
Gria 1	F	60°C	TGTTTGCCTACATTGGAGTGAG	149 pb
	R		GAACCACAGGCTGTTGAATATG	
Grm 1	F	60°C	CTATCATAGCCATCGCCTTTTC	143 pb
	R		GAGGAAAATACCAGCCAGAATG	
RPL19	F	58°C	CTGAAGGTCAAAGGGAATGTG	195 pb
	R		GGACAGAGTCTTGATGATCTC	
Hk2	F	60°C	CTTCTCGTTCCCCTGCCACC	202 pb
	R		CCATGTAGCAGGCGTTGCTG	
PFKm	F	60ºC	GACGTGACCAAGGCTATGGA	313 pb
	R		CAGTCCAGCCTCCAACATAG	
PDK4	F	58ºC	AGTGTGCAAAGATGCTCTGC	132 pb
	R		AGAGCATGTGGTGAAGGTGTG	
PGC 1α	F	60ºC	TCCTCTGACCCCAGAGTCAC	143 pb
	R		CTTGGTTGGCTTTATGAGGAGG	

Cs	F	60ºC	CTCTCTCTCCGATCCCTTCCC
	R		AGGACGAGGCAGGATGAGTTCT
CPT1	F		TGCCTGCCAGTTCCATTAAGC
	R		GTCTCACTCCTCTTGCCAACAG
βHAD	F		CTCCATGTCCTCCTCTTCCTCTGC
	R		CAGCCCGCCGCCGATGAC
PEPCK	F		----------
	R		----------
PKRL	F		----------
	R		----------
G6Pase	F		----------
	R		----------
FAS	F		----------
	R		----------

Bio-Rad Protein Assay: Determinação de proteínas

Diferentes concentrações de BSA (0, 0,25, 0,5, 1, 1,5 e 2 mg/mL) foram obtidas a partir de 10mg/mL de BSA diluído em tampão SDS 1% para a determinação de proteína total. Cada poço foi preenchido com 200 ul Biorad diluído em água (1:5). A placa foi incubada durante 5 até 15 minutos antes da leitura a 595nm num leitor de placas. A proteína detectada foi utilizada para realização do western Blot.

Western Blot

Amostras de proteínas foram preparadas com base em concentrações medidas no BioRad para obter amostras de proteínas com a mesma concentração (10 µg). Em seguida foi adicionado 6 uL de tampão Laemmli 6X. As proteínas foram então transferidas para uma membrana PVDF e incubadas com o tampão de bloqueio (TBS diluída 10X (Euromedex), 0,3% de Tween 20 (Euromedex) e BSA (Euromedex) ou 4% de leite) durante cerca de 2 horas. Após a incubação, com agitação, foi adicionado o anticorpo primário diluído a 1/1000 em tampão de bloqueio durante a noite a 4°C. Diferentes anticorpos secundários foram utilizados para avaliação da expressão de proteínas. Os resultados foram lidos pela câmara ChemiDoc (Biorad).

10. Análises dos dados

Os dados de homogeneidade foram checados pelo teste de Bartlett's. A comparação entre os dados do grupo controle e do grupo experimental foi realizado pelo teste "t" de Student não pareado. Para os experimentos envolvendo desenervação do corpo carotídeo foi utilizado o teste de análise de variância two-way (ANOVA) e o pós-teste de Bonferroni. O nível de significância foi considerado quando $p<0.05$. Os dados foram expressos como média ± epm (erro padrão da média) e análise estatística realizadas no GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA).

6. Resultados

ARTIGO 01 – ARTIGO A SER SUBMETIDO AO NUTRITION REVIEW JOURNAL

Informações da revista: Fator de impacto: 3.4; Qualis A2 – MEDICINA II

6.1 Artigo 01: Association between perinatal malnutrition and development of hypertension: Potential role of the respiratory-sympathetic activity and epigenetic mechanisms

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Abstract

In recent decades it has been well documented that nutritional insults during critical stages of development (pregnancy, lactation and first infancy) induces arterial hypertension in adulthood. The biological phenomenal that link these events is termed as "phenotypic plasticity" and refers to phenotypic flexibility to environmental changes. This plasticity acts as a strategic key to organism survives, initially, but, reflects in late term health problems. Human and animal studies indicate that fetal exposure to an adverse maternal environment, how a protein undernutrition, may alter the morphology and physiology of the cardiovascular, renal, neural systems and contribute to the development of hypertension. Physiological dysfunctions in these systems elicited by protein restriction, can lead to a misbalance of the short- and long-term control of the arterial pressure. Recently, it has been shown that the maternal protein restriction alter the central control of arterial pressure by a mechanism that include enhanced sympathetic-respiratory coupling. Study show that this cenary is early caracterized as higher phrenic burst, frequency and amplitude, leading to increased baseline respiratory frequency and ventilation before the onset of hypertension, which is predominant the increase of cardiovascular tone. Thus, it is well recognized that neurons located in the brainstem, by many pathways, play important role in the onset and progression of hypertension via activation of the respiratory-sympathetic nervous system. One hypothesis is that these dysfunctions result in epigenetic changes, which include acetylation and methylation of DNA and histones modification. Changes in the pattern of methylation imply alteration in the gene expression, resulting in dysfunction in cell development and function and can contribute to the development of arterial hypertension.

Keywords: Fetal programming, hypertension, protein undernutrition, developmental plasticity, respiratory control, sympathetic activity

Introduction

Arterial hypertension (AH) is a major risk factor for cardiovascular dysfunction, recognized as the main cause of morbi-mortality of worldwide (Hedner *et al.*, 2012). Previous reports from World Health Organization (WHO) have shown that AH affects almost one billion people and at least 13-15% of all global deaths is directly caused by raised blood pressure. However, the underlying cause of AH has been difficult to identify due to its multi-factorial nature.

It is now well established and widely accepted that adverse fetal or neonatal environment as protein restriction experienced *in utero* or during perinatal life (gestation, lactation and early infancy) can leading to structural and molecular alteration and predispose to development of AH (Barker e Osmond, 1988; Barker e Law, 1994; Mcmullen e Langley-Evans, 2005; De Brito Alves, Nogueira, *et al.*, 2014b).

The biological phenomena that link events experienced *in utero* with higher predisposing to the development of hypertension and metabolic disease later in life is termed "phenotypic plasticity" and refers to phenotypic flexibility to environmental changes (Barker *et al.*, 2005; West-Eberhard, 2005; Schlichting e Wund, 2014).

Previously, experimental data reported that alteration in the kidney, evidenced by the reduced number of nephron and impaired tubular Na+ reabsorption could, mechanistically, be linked to development of AH in protein-restricted rats during perinatal life (Langley-Evans, 1997; Costa-Silva *et al.*, 2009; Luzardo *et al.*, 2011; Vieira-Filho *et al.*, 2014).

On the other hand, recently it has been suggested new insights into the etiologic mechanisms underlying involved in the development of AH in protein-restricted rats. Recent findings have demonstrated that respiratory-sympathetic overactivation linked to higher peripheral chemosensitivity could be a predisposing factor to development of AH in protein-restricted rats (De Brito Alves, Nogueira, *et al.*, 2014b; Barros *et al.*, 2015; De Brito Alves *et al.*, 2015). In addition, epigenetic mechanism that affect the gene expression have been proposed to mediate the effects of maternal dietary on hypertension susceptibility in the offspring (Goyal *et al.*, 2010; Fernandez-Twinn *et al.*, 2015; Joss-Moore *et al.*, 2015).

In order to help and highlight these new insights, in the present review, we will discuss the potential role of respiratory-sympathetic overactivity and epigenetic alteration as potential mechanisms between malnutrition during pregnancy and lactation and development of AH in offspring adult life.

Evidences between maternal protein restriction and hypertension

Some of the initial indications of a relationship between the intrauterine environment and later health/disease status occurred during Dutch Famine in World War II (1945-1946) (Barker, 1990; Stein *et al.*, 2006). The famine of approximately 6 months, it affected pregnant women and babies. The babies exposed to famine *in utero* were associated with higher prevalence of hypertension, type 2 diabetes and coronary heart disease in adult life (Stein *et al.*, 2006).

Then, others epidemiological studies also have shown that intrauterine environment has a strong association with development of hypertension in the offspring. For example, forty years after fetal exposure to famine afflicted Biafra famine during the *Nigerian civil war* (1967-1970) adults exhibited higher levels in systolic arterial pressure (+7 mmHg) and diastolic arterial pressure (+5 mmHg) . Demonstrating that fetal and infant undernutrition were associated with significantly increased risk of hypertension in 40-year-old Nigerians (Hult *et al.*, 2010). In addition, a retrospective study with 12.065 subject born during "*The Great Chinese Famine*" demonstrated that the hypertension risk in adult life was 1.36 fold higher in those exposed to famine during the first half of pregnancy (Wang *et al.*, 2012).

Recently, a pilot study performed with 70 European Jews born in countries under Nazi rule during holocaust period (1940-1945) revealed that the prevalence of systemic arterial hypertension was significantly higher (62.9% vs. 43%, P = 0.003) when compared to 230 Israeli-born individuals of the same descent, age, and gender, extracted from the Israel National Health Interview Survey-2 (Bercovich *et al.*, 2014). These findings clearly demonstrate that there is a strong relationship between maternal malnutrition and higher susceptibility to the development of hypertension in the offspring.

Maternal low-protein diet during gestation and/or lactation is one of the most extensively studied animal models recapitulating phenotypic plasticity and developmental origin of AH (Langley-Evans, 1997; Mcmullen e Langley-Evans, 2005; De Brito Alves, Nogueira, *et al.*, 2014b). Feeding a low-protein diet (8% protein) during gestation and lactation followed by the consumption of a normocaloric diet is associated with long-lasting growth restriction and hypertension, even when the offspring is weaned on a control diet (Langley-Evans, 1997; Costa-Silva *et al.*, 2009; Barros *et al.*, 2015; De Brito Alves *et al.*, 2015; Langley-Evans, 2015).

Kidney, play a major role in the long-term control of arterial blood pressure by regulating Na^+ intake/excretion and experimental findings suggest an association between nephron number and birthweight, demonstrating that nephron number is extremely sensitive

to maternal undernutrition and can be constrained by food restriction or protein restriction (Langley-Evans *et al.*, 1996; Langley-Evans, 2015).

In this context, it has been reported that offspring from dams subjected to maternal protein restriction during pregnancy and/or lactation exhibit an decrease in nephron number (Hughson *et al.*, 2003; Zandi-Nejad *et al.*, 2006), impaired tubular Na^+ reabsorption (Burnier *et al.*, 1994; Costa-Silva *et al.*, 2009), increase in mRNA expression of (Na^++K^+) ATPase in proximal tubules (Luzardo *et al.*, 2011; Vieira-Filho *et al.*, 2011), transcriptional up-regulation and protein expression of Na^+ transporters (Manning *et al.*, 2002), contributing to the programming of hypertension.

Although there is evidence demonstrating that renal damage induce hypertension in protein-restricted rats during pregnancy and/or lactation, the mechanisms that alters the renal functionality are yet poorly understood.

Maternal protein-restriction and the development of hypertension: new mechanistic consideration

Although several studies have shown that maternal protein restriction leads to the development of AH in male offspring, the underlying mechanisms that predispose hypertension are very complex and not fully understood. The regulation of arterial pressure (AP) is one of the most complex physiological functions and depends on integrated actions of cardiovascular, renal, neural and endocrine systems, which all act on different time scales and with different means of control (Corry e Tuck, 1999). In this context, it is well described the important role of different controller, including the autonomic nervous system, balancing the sympathovagal activity and peripheral sensors, where it is highlighted the influence of arterial baroreceptors, chemoreceptors, and renal system (Krieger *et al.*, 1982; Lohmeier *et al.*, 2015). However, physiological dysfunctions in these systems, such as those elicited by protein restriction, can lead to a misbalance of the short- and long-term control of the AP and the development of the systemic AH.

Clinical and experimental data involving hypertension models demonstrated that sympathetic-respiratory overactivation linked to higher central-peripheral chemosensitivty play a key role in the development of hypertension (Zoccal *et al.*, 2007; Costa-Silva *et al.*, 2010; Zoccal e Machado, 2011; Costa-Silva *et al.*, 2012; Krieger *et al.*, 2014; Moraes, Bonagamba, *et al.*, 2014).

Indirect measurement of autonomic status might be performed though of study of variability of systolic arterial pressure (SAP) and heart rate (HR). High-frequency (HF)

oscillation is related to respiration and associated with cardiac vagal modulation; low-frequency (LF) oscillation is related to cardiac and vasomotor sympathetic modulation to heart and blood vessels (Heart rate variability: standards of measurement, physiological interpretation and clinical use. Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). In addition, pharmacologic blockage have been used for evaluate sympathetic and vagal activity in clinical and experimental studies.

Convincingly, our research group have shown that rats exposed to protein restriction during critical periods (pregnancy and lactation) exhibit augmented sympathetic tone and hypertension at 90th days old (De Brito Alves, Nogueira, *et al.*, 2014b; Barros *et al.*, 2015). This autonomic unbalance in protein-restricted rats was supported by the increase in LF oscillation of the SAP and HR, which is related to enhanced vasomotor and cardiac sympathetic modulation to blood vessels and heart, as well as by the larger sympathetic tone to the heart and cardiovascular system, observed by pharmacological tools (Barros *et al.*, 2015). Taken all together, these findings suggest that the hyperactivity of the sympathetic nervous system may be associated to hypertension development in protein-restricted adult rats. In addition to these experimental evidences, Franco et al. (2008) showed that 66 children (8- to 13-year-old) that born at term but with a small birthweight for gestational age, exhibited augmented circulating noradrenaline levels, angiotensin II and higher angiotensin-converting enzyme (ACE) activity when compared those born at term with an appropriate birthweight (Franco *et al.*, 2008). Suggesting that restricted fetal growth might affect sympathoadrenal and renin-angiotensin systems and predispose the development of hypertension.

In order to better understanding as maternal protein restriction during pregnancy and lactation predispose development of hypertension in adult life, studies have been conducted for investigating possible alteration in respiratory-sympathetic coupling in the offspring of protein-restricted dams before the onset of hypertension. Thus, it is already described that maternal protein restriction during pregnancy and lactation leads to relevant short-term effects on respiratory function of the offspring, such as higher phrenic burst, frequency and amplitude, leading to increased baseline respiratory frequency (up to 28 %) and ventilation (up to 40 %) before the onset of hypertension (De Brito Alves, Nogueira, *et al.*, 2014b; De Brito Alves *et al.*, 2015). In addition, using *in situ* preparation it was demonstrated that these respiratory dysfunctions were associated with enhanced baseline sympathetic activity and amplified ventilatory and sympatho-excitory responses to peripheral chemoreflex activation prior to establishment of hypertension (De Brito Alves *et al.*, 2015), which apparently are

linked with a high levels of hypoxic inducible factor (HIF-1α) in carotid body peripheral chemoreceptors (De Brito Alves *et al.*, 2015). In this way, clinical studies also have shown that activity of peripheral chemoreceptors it is augmented in preterm infants (Katz-Salamon e Lagercrantz, 1994; Martin *et al.*, 2012), demonstrating that oxygen-sensitive peripheral chemoreceptor activity is progressively increased over the first 4 weeks of postnatal life and has been associated with a higher incidence of apnea in preterm neonates (Stephan-Blanchard *et al.*, 2010; Martin *et al.*, 2012). In all, these data support the hypothesis that alterations in respiratory network and respiratory chemosensitivity may contribute to the higher levels of sympathetic nerve discharges in protein-restricted rats, which in part could contribute to the higher activation of the renin-angiotensin-aldosterone (RAAS), arginine-vasopressin (AVP) systems in kidney (Cornock *et al.*, 2010; Langley-Evans, 2015), increase in the cardiovascular sympathetic tone (Barros *et al.*, 2015) and hypertension (De Brito Alves, Nogueira, *et al.*, 2014b) evidenced in the adult rats.

Furthermore, it is well recognized that neurons located in the brainstem, by many pathways, play important role in the onset and progression of hypertension via activation of the respiratory-sympathetic nervous system (De Kloet *et al.*, 2015), mainly presympathetic neurons identified within the rostral ventrolateral medulla (RVLM), which have been associated with sympathetic overactivity and development of AH (Zoccal *et al.*, 2009; Moraes, Machado, *et al.*, 2014).

On the other hand, reactive oxygen species (ROS) into brainstem also have a potential role in modulate sympathetic activity and AP in spontaneously hypertensive rats (SHR), suggesting that oxidative stress can contribute to higher sympathetic activity and AH (Chan *et al.*, 2006; Hirooka *et al.*, 2010; Nishihara, Hirooka, Kishi, *et al.*, 2012). Experimental findings from Ferreira and colleagues (2015) demonstrated that maternal protein restriction during pregnancy and lactation alters brainstem antioxidant metabolism in adult offspring, characterized by the increase of lipid peroxidation linked to down-regulation of superoxide dismutase (SOD), catalase and glutathione peroxidase activities (Ferreira *et al.*, 2015). Thus, it is possible that central oxidative imbalance, in part, play a key role in increase of sympathetic activity and development of AH in protein-restricted rats.

In addition, oxidative stress module the balance between GABA/glutamate in the RVLM (Chan e Chan, 2014). ROS in the RVLM enhances glutamatergic neurotransmission, probably by the MAPK signaling pathways and attenuates GABAergic inhibitory inputs to the RVLM (Chan *et al.*, 2003). The excitatory amino acid (EAA) glutamate has been demonstrated to play an important role in cardiovascular regulation, leading to higher

sympathoexcitatory inputs to RVLM neurons (Costa-Silva *et al.*, 2010; Moraes *et al.*, 2011). Thus, we hypothesized that enhanced glutamatergic transmission in RVLM may be important for respiratory-sympathetic overactivity and hypertension in rats subjected to perinatal protein restriction.

Previous reports have shown that an augmentation of ROS dependent of NADPH oxidase activation in brain leads to a long-term pressor response to angiotensin II via transcriptional up-regulation of angiotensin II type-1 (AT1) receptors mRNA expression, indicating that brain renin-angiotensin system are important to regulate AP and sympathetic activity (Dai *et al.*, 2015; Wang, L. H. *et al.*, 2015). In this context, it was shown that maternal low protein diet during second half of the pregnancy promoted increase in mRNA expression of angiotensinogen and angiotensin converting enzyme-1 (ACE-1) associated with a decrease in mRNA levels of angiotensin II type-2 (AT2) receptors in the fetal brain (Goyal *et al.*, 2010). This alteration was linked to hypomethylation of the CpG islands in the promoter regions of ACE-1 gene (Goyal *et al.*, 2010), suggesting that epigenetic mechanism could be involved in development of sympathetic overactivity-induced hypertension in perinatal protein-restricted rats.

Epigenetic pathways and hypertension

Altered gene expression patterns caused by poor perinatal nutritional conditions and their persistence through adult life might be mediated by epigenetic events, and have been invoked as a developmental and nutritional programming mechanism, which predisposes to the late emergence of metabolic diseases (Gabory *et al.*, 2011; O'sullivan *et al.*, 2012). The main epigenetic features of mammalian cells include DNA methylation, posttranslational histone modifications and RNA-based mechanisms (miRNAs) (Zampieri *et al.*, 2015). The epigenetic mechanisms involved, including trans-generational transmission of methylation histone marks, mediated by the polycomb repressive complex, have recently been elucidated (Ozanne, 2015). Thus, gene expression dysregulation and harmful epigenetic mechanisms can potentially contribute to the development of AH (Wang, J. *et al.*, 2015). An important aspect to consider is the reversibility of these epigenetic mechanisms, which potentially are reversible and may be counteracted by interventional measures that aim to normalize the effects caused by poor nutritional conditions experienced during intra-uterine life.

DNA methylation is the first epigenetic alteration identified on DNA and can be determined during fetal development, which plays an important role in chromatin organization and gene expression (Miranda e Jones, 2007). The methylation reaction is

characterized by insertion of methyl group deriving from S-adenosyl-methionine onto the C5 position of cytosine residue, catalyzed by the enzymes DNA methytransferases (DNMTs) (Miranda e Jones, 2007; Zampieri *et al.*, 2015).

DNA methylation (hypermethylation) can silence genes through a process that leads to the alteration of chromatin structure (Miranda e Jones, 2007). Interestingly, it was reported that hypomethylation mechanisms could leads to higher ACE, β-adrenergic receptors and Na^+ channels expression in hypertensive condition (Riviere *et al.*, 2011; Friso *et al.*, 2015; Zampieri *et al.*, 2015).

In this way, Rangel and colleagues (2014) demonstrated that the mean DNA methylation at individual CpG sites within the ACE gene promoter was significantly lower in low birth weight (LBW) children at 6 to 12 year-old (Rangel *et al.*, 2014). This alteration in methylation levels it was associated to higher ACE activity and AP in LBW children (Rangel *et al.*, 2014).

On the other hand, it was verified that the expression of HIF-1α is enhanced in the carotid bodies (De Brito Alves *et al.*, 2015), heart (Ito *et al.*, 2011) and brain (Ito *et al.*, 2012) in rats from dams exposed to protein restriction during pregnancy and/or lactation. The HIF-1α is related to the response of cell to reduced oxygen (hypoxia) and its higher expression is associated with increased risk of hypertension and enhanced sensory activity of peripheral chemoreceptors (Prabhakar, 2013). The underlying mechanism involved in the HIF-1α up-regulation in protein-restricted rats is still unclear, but we theorized that DNA metylation mechanism could be involved in the enhanced expression of HIF-1α. In light of this evidence, hypomethylation profile may be the key mechanism in the development of sympathetic hyperactivity, increased respiratory chemosensitivity and hypertension (**Figure 1**). Thus, futures studies on epigenetic modulations could help us to better understand the mechanisms involved in development of programming hypertension.

Conclusion

Nutritional insults during pregnancy, lactation and early infancy represent a determinant factor in the health-disease process of individual. To understand this process involved in early-life programming of arterial hypertension is starting point to development of strategies that aim to prevent and reverse this pathology. Thus, it possible to encourage parents to adopt lifestyle changes that could have health implications and to decrease significantly the number of cardiovascular diseases.

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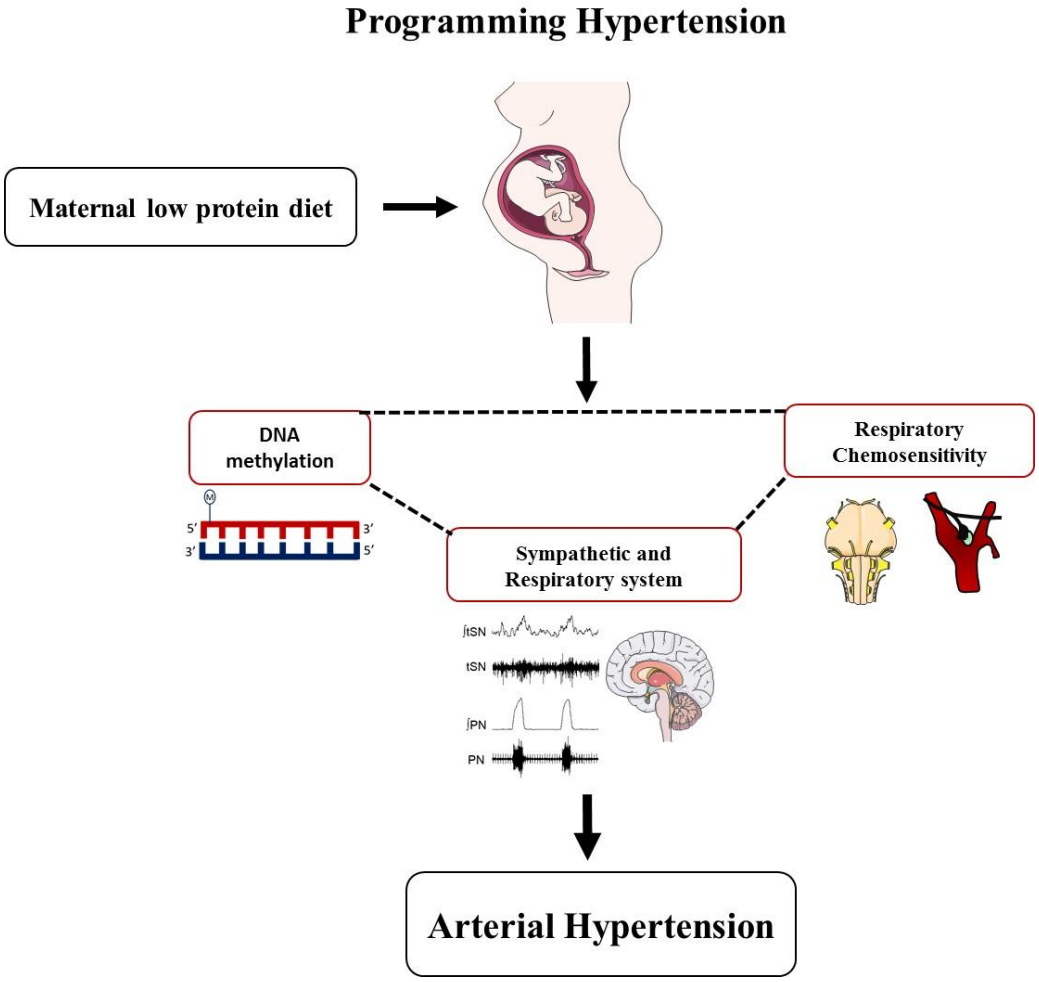
Programming Hypertension
Maternal low protein diet
DNA methylation
Respiratory Chemosensitivity
Sympathetic and Respiratory system
∫tSN
tSN
∫PN
PN
Arterial Hypertension

Figure 1.

ARTIGO 02 – ARTIGO PUBLICADO NO THE JORUNAL OF NUTRITION

ARTIGO 02: Maternal Protein Restriction Increases Respiratory and Sympathetic Activities and Sensitizes Peripheral Chemoreflex in Male Rat Offspring

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Maternal Protein Restriction Increases Respiratory and Sympathetic Activities and Sensitizes Peripheral Chemoreflex in Male Rat Offspring[1–3]

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Abstract

Background: Maternal protein restriction in rats increases the risk of adult offspring arterial hypertension through unknown mechanisms.

Objectives: The aims of the study were to evaluate the effects of a low-protein (LP) diet during pregnancy and lactation on baseline sympathetic and respiratory activities and peripheral chemoreflex sensitivity in the rat offspring.

Methods: Wistar rat dams were fed a control [normal-protein (NP); 17% protein] or an LP (8% protein) diet during pregnancy and lactation, and their male offspring were studied at 30 d of age. Direct measurements of baseline arterial blood pressure (ABP), heart rate (HR), and respiratory frequency (Rf) as well as peripheral chemoreflex activation (potassium cyanide: 0.04%) were recorded in pups while they were awake. In addition, recordings of the phrenic nerve (PN) and thoracic sympathetic nerve (tSN) activities were obtained from the in situ preparations. Hypoxia-inducible factor 1α (HIF-1α) expression was also evaluated in carotid bifurcation through a Western blotting assay.

Results: At 30 d of age, unanesthetized LP rats exhibited enhanced resting Rf ($P = 0.001$) and similar ABP and HR compared with the NP rats. Despite their similar baseline ABP values, LP rats exhibited augmented low-frequency variability (~91%; $P = 0.01$). In addition, the unanesthetized LP rats showed enhanced pressor ($P = 0.01$) and tachypnoeic ($P = 0.03$) responses to peripheral chemoreflex activation. The LP rats displayed elevated baseline tSN activity (~86%; $P = 0.02$) and PN burst frequency (45%; $P = 0.01$) and amplitude (53%; $P = 0.001$) as well as augmented sympathetic ($P = 0.01$) and phrenic ($P = 0.04$) excitatory responses to peripheral chemoreflex activation compared with the NP group. Furthermore, LP rats showed an increase of ~100% in HIF-1α protein density in carotid bifurcation compared with NP rats.

Conclusion: Sympathetic-respiratory overactivity and amplified peripheral chemoreceptor responses, potentially through HIF-1α–dependent mechanisms, precede the onset of hypertension in juvenile rats exposed to protein undernutrition during gestation and lactation. *J Nutr* 2015;145:907–14.

Keywords: protein undernutrition, hypertension, sympathetic overactivity, peripheral chemoreflex, hypoxia-inducible factor 1 α

Introduction

Arterial hypertension is a major risk factor for cardiovascular dysfunction, which affects almost 1 billion people and is recognized as a major cause of morbidity and mortality worldwide (1). However, the underlying cause of hypertension has been difficult to identify due to its multifactorial nature.

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[3] Supplemental Figures 1 and 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online contents at http://jn.nutrition.org.
[9] DBZ and JHC-S contributed equally to this work.

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Hypertension may arise from a combination of genetic factors and lifestyle-related behaviors (1). In addition, adverse events experienced in utero or during perinatal life (gestation, lactation, and early infancy) can affect the development of physiologic systems, leading to increased risk of hypertension and metabolic diseases later in life (2, 3).

For example, maternal undernutrition has been associated with low nephron number, kidney disease, insulin resistance, and obesity because of rapid weight gain in childhood or adolescence (4, 5). The biological phenomenon underlying these associations is known as phenotypic plasticity, which refers to the ability of a single genotype to produce variable behavioral, morphologic, and/or physiologic phenotypes (6) in individuals in response to different environmental circumstances encountered during development.

The offspring of rat dams subjected to a maternal low-protein (LP)[10] diet is a model that is often used to study the mechanisms of maternal undernutrition–related hypertension (6–8), which has been suggested to be associated with changes in the functioning of the sympathetic nervous system (9, 10). However, there is no direct evidence demonstrating that sympathetic vasoconstrictor tonus is elevated in rats subjected to a perinatal LP diet.

We recently showed that juvenile rats subjected to protein undernutrition during gestation and lactation exhibit increased baseline respiratory frequency associated with amplified ventilatory responses to hypoxia and hypercapnia (7). These findings indicated that the functioning of the respiratory network, in addition to that of the sympathetic nervous system, is affected by an LP diet during gestation and lactation, likely through a common excitation mechanism. In this regard, afferent inputs from peripheral chemoreceptors to the central nervous system, which are mainly generated in response to hypoxic stimuli, evoke reflex responses of sympatho-excitation and tachypnoea (11). Therefore, changes in the functioning of the peripheral chemoreflex may be involved in the exaggerated sympathetic and respiratory responses observed in the offspring of protein-restricted rats.

It has been shown that the sensitization of peripheral chemoreceptors is a risk factor for sympathetic overactivity and the development of arterial hypertension (12, 13). An important molecular mechanism involved in the enhanced sensory activity of peripheral chemoreceptors is the activation of hypoxia-inducible factor (HIF) (14, 15). Indeed, there is evidence that high expression of HIF-1α during early life is associated with an increased risk of developing hypertension (16, 17).

In this context, in the present study, we hypothesized that juvenile rats from dams subjected to protein undernutrition during pregnancy and lactation would exhibit enhanced baseline sympathetic and inspiratory motor activities associated with amplified respiratory and sympathetic responses to peripheral chemoreflex activation and enhanced HIF-1α concentrations in the carotid body peripheral chemoreceptors. This hypothesis was investigated in the offspring of protein-restricted dams before the onset of hypertension (7) to verify whether these changes are the cause or consequence of an increase in arterial pressure.

[10] Abbreviations used: HF, high frequency; HIF, hypoxia-inducible factor; HR, heart rate; KCN, potassium cyanide; LF, low frequency; LP, low protein; MAP, mean arterial pressure; NP, normal protein; PN, phrenic nerve; Rf, respiratory frequency; SAP, systolic arterial pressure; tSN, thoracic sympathetic nerve.

Methods

The experimental protocol was approved by the Ethical Committee of the Biological Sciences Center (protocol 044454/2010–94) at the Federal University of Pernambuco and by the Animal Experimentation Ethics Committee of the School of Dentistry of Araraquara at São Paulo State University (protocol 21/2012), Brazil. All efforts were made to minimize animal discomfort and the number of rats used; in addition, we followed the Guidelines for the Care and Use of Laboratory Animals.

Animals and experimental groups. Virgin female albino Wistar rats (*Rattus norvegicus*) were obtained from the Academic Center of Vitoria de Santo Antão, Federal University of Pernambuco, Brazil. The rats were maintained in a room with a temperature of 22 ± 1°C and a controlled light-dark cycle (dark: 1800–0600 h). Standard laboratory feed pellets (52% carbohydrate, 21% protein, and 4% lipids, Labina; Purina Agriband) and water were consumed ad libitum up to the 3-mo point, when the rats were mated (2 females for 1 male). The day on which spermatozoa were identified in a vaginal smear was considered the date of conception, and pregnant rats were transferred to individual cages. Two experimental groups were designated according to diet manipulation: dams fed a 17% casein diet [normal-protein (NP) group; $n = 6$] and dams fed a 8% casein diet (LP group; $n = 6$) and water ad libitum.

The diets were mixed at the Laboratory of Experimental Nutrition–Academic Center of Vitoria de Santo Antão, Federal University of Pernambuco, according to the American Institute of Nutrition–AIN-93 diet (18, 19). The casein was previously analyzed and found to be 85% pure (85 g of protein for each 100 g of casein). The diets were isoenergetic and were fed during pregnancy and lactation. Both diets presented the same amount of vitamin and mineral mix. Only the amount of protein and carbohydrate was changed (18). Offspring were standardized as litters of 8 pups 48 h after birth. Male offspring were used in each litter and females were used only to standardize the size of each litter to 8 pups (7). At weaning, 3 or 4 male offspring from each litter were randomly housed in collective cages and received a standard diet ad libitum. At least 2 or 3 male offspring from each litter were used to compose the NP or LP groups and to perform the experimental protocol. All of the experiments were performed in 30-d-old juvenile rats.

Cardiovascular and respiratory evaluations in vivo. One day before the experiments, the NP ($n = 8$) and LP ($n = 11$) rats were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg), and the femoral artery and vein were cannulated [Polyethylene Tubing (PE)-50 connected to PE-10; Clay Adams]. The catheters were filled with heparinized saline (NaCl 0.9%), tunneled subcutaneously, and exteriorized through the back of the neck. After surgery, the rats were administered an injection of ketoprofen (5 mg/kg, intraperitoneally), and a period of 24 h was allowed to pass until the rats had fully recovered from the surgical and anesthetic procedures. The next day, the mean arterial pressure (MAP) and heart rate (HR) of unanesthetized freely moving rats were recorded by connecting the arterial catheter to a pressure transducer. The signals were amplified (ML866/P, Power Lab; ADInstruments), sampled at 2 kHz, and digitalized by using appropriate software (LabChart7 Pro; ADInstruments). Recordings of the baseline pulsatile arterial pressure, MAP, and HR were made for 30–50 min. After 50 min of acclimatization and cardiovascular recordings, measurements of the respiratory frequency (Rf) were also performed by using the whole-body plethysmography method (20). Before recording baseline data, the rats were placed into a Plexiglas chamber (5 L) that was flushed with humidified room air and maintained at a temperature of 25°C. After this acclimatization period, the Rf was recorded as the airflow was suspended for short periods (3 min), and the pressure oscillations caused by breathing were captured by a pressure differential transducer connected to a signal amplifier (ML141 Spirometer, PowerLab; ADInstruments). The signals were then captured by an acquisition system and data analysis was performed (PowerLab; ADInstruments). All of the data were analyzed offline with the use of appropriate software (LabChart 7 Pro; ADInstruments).

After the baseline recordings of Rf and arterial pressure, the peripheral chemoreflex was activated through the intravenous injection of potassium cyanide (KCN; 40 μg/100 μL per rat; Merck) in accordance with previous reports (11, 21). At the end of the experiments,

the rats were killed with a 1-mL overdose of a mixture of ketamine (80 mg/kg, intraperitoneally) and xylazine (10 mg/kg, intraperitoneally).

An indirect evaluation of the autonomic modulation of vascular resistance and cardiac function was performed through an analysis of the variability in the arterial pressure and HR in the frequency domain (22). Oscillations of arterial pressure and HR at the low-frequency (LF) range are representative of the modulatory effects of sympathetic activity controlling vascular tonus and heart activity, whereas oscillations at the high-frequency (HF) range are associated with a respiratory or parasympathetic modulation of blood vessels or the heart, respectively (22–24). To reach this goal, a beat-by-beat time series of the systolic arterial pressure (SAP) and HR were extracted from the baseline cardiovascular recordings (10-min epochs) of the pulsatile arterial pressure of the NP and LP rats (Chart Pro; ADInstruments), and the overall variability of these series was assessed through fast Fourier transformation infrared spectroscopy (Cardioseries software, version 2.4) (25). The power of the oscillatory components obtained from the rats belonging to the NP and LP groups was quantified in 2 frequency bands: LF (0.20–0.75 Hz) and HF (0.75–3.0 Hz) (22, 26).

In situ working heart-brainstem preparation. Juvenile rats at 30 d of age [NP (n = 6) and LP (n = 8)] were deeply anesthetized with halothane (Astra Zeneca), such that the withdrawal responses to noxious pinching of the tail and paw were absent. The rats were then transected caudally to the diaphragm and submerged in cooled Ringer solution (in mM: 125 NaCl, 24 $NaHCO_3$, 3 KCl, 2.5 $CaCl_2$, 1.25 $MgSO_4$, 1.25 KH_2PO_4, and 10 dextrose). They were made insentient by decerebration at the precollicular level, skinned, and had the descending aorta isolated. Preparations were then transferred to a recording chamber, where the descending aorta was cannulated and perfused retrogradely with modified Ringer solution containing lactate (2 mM), an oncotic agent (1.25% polyethylene glycol; Sigma), and a neuromuscular blocker (vecuronium bromide, 3–4 μg/mL; Cristalia) by using a roller pump (Watson-Marlow 502s) via a double-lumen cannula. Perfusion pressure was maintained in the range of 50–70 mm Hg by adjusting the rate flow between 21 and 25 mL/min and by adding vasopressin to the perfusate (6–12 nM; Sigma), as previously described (27). Electrical activity in all nerves was recorded by using glass suction bipolar electrodes held by a micromanipulator (Narishige). Left phrenic nerve (PN) discharges were recorded from the central end and its rhythmic ramping activity gave a continuous physiologic index of preparation viability. Thoracic sympathetic nerve (tSN) activity was recorded from the thoracic sympathetic chain at the level of T10–T12. All of the signals were amplified, band-pass filtered (0.05–5 kHz), and acquired with an A/D converter (CED 1401; Cambridge Electronic Design) on a computer using Spike2 software (version 7; Cambridge Electronic Design). Peripheral chemoreceptors were stimulated by injections of KCN (0.05%, 50 μL) into the descending aorta of the working heart-brainstem preparation via the perfusion cannula, as previously described (28).

All of the analyses of rectified and integrated (50-ms) signals were performed off-line by using the Spike 2 software with custom-written scripts. Before analyses, PN and tSN recordings were subtracted from the electrical noise obtained after the death of the working heart-brainstem preparation (induced by turning the pump off). For baseline measurements, PN activity was assessed by its frequency (cycles per minute), amplitude (μV), burst duration (inspiratory time, s), and burst interval (expiratory time, s). tSN activity was assessed by its mean activity (μV) and by the amplitude of inspiratory-related bursts (μV), which was calculated by the value difference between the maximal and lowest activity observed during inspiratory and postinspiratory phases. With respect to the changes induced by peripheral chemoreflex activation, the phrenic frequency reflex response was assessed by the difference between the baseline frequency and the peak of response observed after the stimulus (ΔPN; expressed in cycles per minute). The sympathetic response was assessed by the measurement of the AUC and expressed as percentage change (ΔtSN; in %) in relation to the activity before the stimulus.

Evaluation of HIF-1α protein density. Under normoxic conditions, separate groups of NP (n = 6) and LP (n = 7) rats that were not subjected to any surgical procedure were killed by an overdose of ketamine (80 mg/kg, intraperitoneally) and xylazine (10 mg/kg, intraperitoneally) for collection of the carotid bifurcation at 30 d of age. The tissues were flash-frozen in liquid nitrogen and stored at −80°C until use. The carotid bifurcation samples were pooled respectively to obtain a sufficient amount of protein. The samples were then sonicated, and protein extracts were obtained in radioimmunoprecipitation assay buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1% SDS, 0.5% sodium deoxycholate, 0.5% NP-40) with protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Protein concentration was determined by using the Bradford method (Bio-Rad Laboratories). Ninety micrograms of protein was submitted to 8% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (GE HealthCare). The membranes were blocked for 1 h by using Tris-buffered saline containing 10% Tween 20 (TBS-T) and 5% (wt: vol) nonfat dry milk (blocking buffer). Antibodies against HIF-1α (mouse monoclonal H1α67, GR45835-1; AbCam) and GAPDH (2118S; Cell Signaling) were used. The antibody against HIF-1α was used diluted 1:500 in TBS-T with 5% of BSA (Sigma-Aldrich). Anti-mouse secondary antibody was diluted 1:5000 in blocking buffer. Blots were developed by using the chemiluminescent ECL Western Blotting System

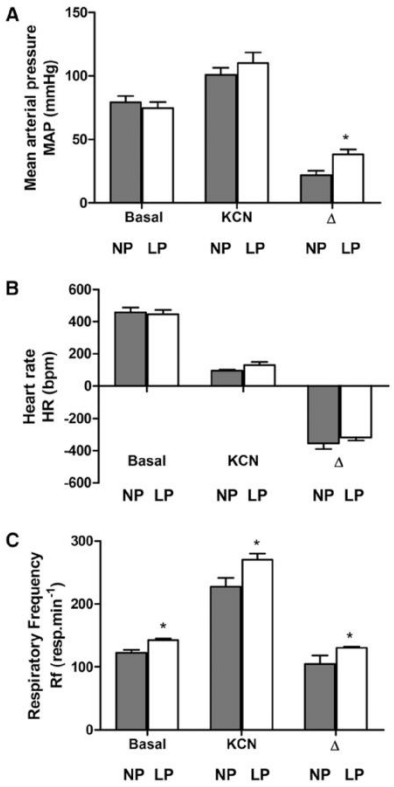

FIGURE 1 MAP (A), HR (B), and Rf (C) of 30-d-old male rat pups of dams fed an NP or an LP diet during pregnancy and lactation. Values are means ± SEMs, n = 8–11. *Different from NP, $P < 0.05$ (unpaired Student's t test). bpm, beats per minute; HR, heart rate, KCN, potassium cyanide; LP, offspring of experimental rat dams fed a low-protein diet (8% protein); MAP, mean arterial pressure; NP, offspring of control rat dams fed a normoproteic diet (17% protein); resp, respirations; Rf, respiratory frequency.

(GE HealthCare). The bands were quantified by densitometry with the use of Image J software (NIH; http://rsbweb.nih.gov/ij/), and the relative densities of HIF-1α were normalized by their respective controls.

Statistical analysis. Each experimental group included at least 2 rats from each litter. Bartlett's test was performed to evaluate data homogeneity of the respiratory and sympathetic variables, and statistical results supported the use of a parametric test. Thus, the significance of the difference between groups was assessed by unpaired Student's *t* test. The significance level was fixed to $P < 0.05$. The data are expressed as means with associated SEs. Statistical analysis was performed by using GraphPad Prism 5.0 software.

Results

Ponderal gain. Pups from mothers subjected to an LP diet had lower birth weight than did the NP group (NP vs. LP: 6.3 ± 0.1 vs. 5.3 ± 0.2 g; $P = 0.04$). Furthermore, the reduced body weight of the LP group was maintained until 30 d of age (NP vs. LP: 86.3 ± 2.9 vs. 62.5 ± 2.6 g; $P = 0.01$).

Conscious rats. Representative baseline and chemoreflex-evoked changes in MAP, HR, and Rf of unanesthetized rats at 30 d of age are shown in **Supplemental Figures 1** and **2.** The baseline MAP ($P = 0.83$; **Figure 1**A) and HR ($P = 0.95$; Figure 1B) were similar between LP and NP rats. However, baseline Rf was higher in the LP group than in the NP group ($P = 0.001$; Figure 1C). Despite the similar baseline values, the autonomic modulation of arterial pressure and HR was altered in LP rats. As can be observed in the representative spectra of SAP (**Figure 2**A), LP rats exhibited an augmented magnitude of oscillation at the LF range ($P = 0.01$; Figure 2B) but not at the HF range ($P = 0.75$; Figure 2C) compared with NP rats. In relation to pulse interval, the LF:HF ratio (an index of sympathetic:parasympathetic balance to the heart) was enhanced in the LP group ($P = 0.001$; Figure 2D).

Peripheral chemoreflex activation with KCN (intravenous) produced pressor, bradycardic, and tachypnoeic responses in both NP and LP groups. The increase in MAP ($P = 0.01$; Figure 1A) and Rf ($P = 0.03$; Figure 1C) were significantly greater in the LP group than in the NP group. In contrast, the magnitude of decrease in HR of both groups were similar ($P = 0.32$; Figure 1B).

tSN and PN activities in situ. Baseline recordings of the PN and tSN activities of representative 30-d-old rats are shown in **Figure 3**A. The LP rats presented higher levels of tSN amplitude than did NP rats ($P = 0.02$; Figure 3B). Average tSN levels were not significantly different between groups ($P = 0.35$; Figure 3C). Furthermore, LP rats exhibited a larger PN burst frequency ($P = 0.01$; Figure 3E) and amplitude ($P = 0.001$; Figure 3D) in comparison to NP rats. The LP rats also showed shorter inspiratory (NP vs. LP: 1.4 ± 0.1 vs. 1.1 ± 0.1 s; $P = 0.04$) and expiratory (NP vs. LP: 4.3 ± 0.6 vs. LP: 2.6 ± 0.2 s; $P = 0.01$) times compared with the NP group. Together, these findings showed augmented sympathetic and inspiratory motor activities for LP rats at baseline conditions.

The activation of peripheral chemoreceptors elicited responses of sympatho-excitation and increased PN burst frequency in the in situ preparations of 30-d-old rats from the NP and LP groups, as shown in **Figure 4**A. We verified that LP rats exhibited greater increases in PN burst frequency ($P = 0.04$; Figure 4B) and higher sympatho-excitatory responses ($P = 0.01$; Figure 4C) than did NP rats.

HIF-1α expression in carotid bifurcation. Bands corresponding to HIF-1α were observed at 120 kDa in samples of carotid bifurcations from NP and LP rats. HIF-1α protein density, which was normalized by GAPDH density, was 119% higher (NP vs. LP: 0.30 vs. 0.67 arbitrary units) in carotid bifurcation samples from LP rats compared with the samples from NP rats, as shown in **Figure 5**.

Discussion

In the present study, we investigated the effects of perinatal protein restriction on baseline and chemoreflex-evoked control of arterial pressure, Rf, sympathetic and phrenic activities, and HIF-1α expression in the carotid bodies. The main findings of this study showed that protein restriction during perinatal development produced in 30-d-old rats *1*) increased baseline

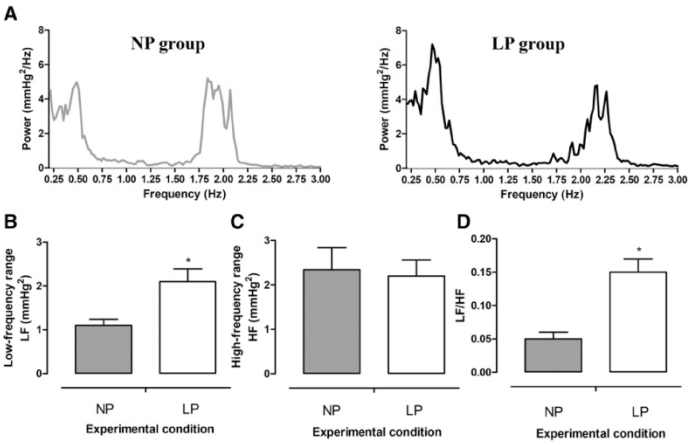

FIGURE 2 Representative spectra of SAP (A), average magnitudes of LF (B) and HF (C) components of SAP, and the LF/HF index of PIs (D) of 30-d-old male rat pups of dams fed an NP or an LP diet during pregnancy and lactation. Values are means ± SEMs, n = 8–11. *Different from NP, $P < 0.05$ (unpaired Student's t test). HF, high-frequency band; LF, low-frequency band; LF/HF, index of sympathetic/parasympathetic balance to the heart; LP, offspring of experimental rat dams fed a low-protein diet (8% protein); NP, offspring of control rat dams fed a normoproteic diet (17% protein); PI, pulse interval; SAP, systolic arterial pressure.

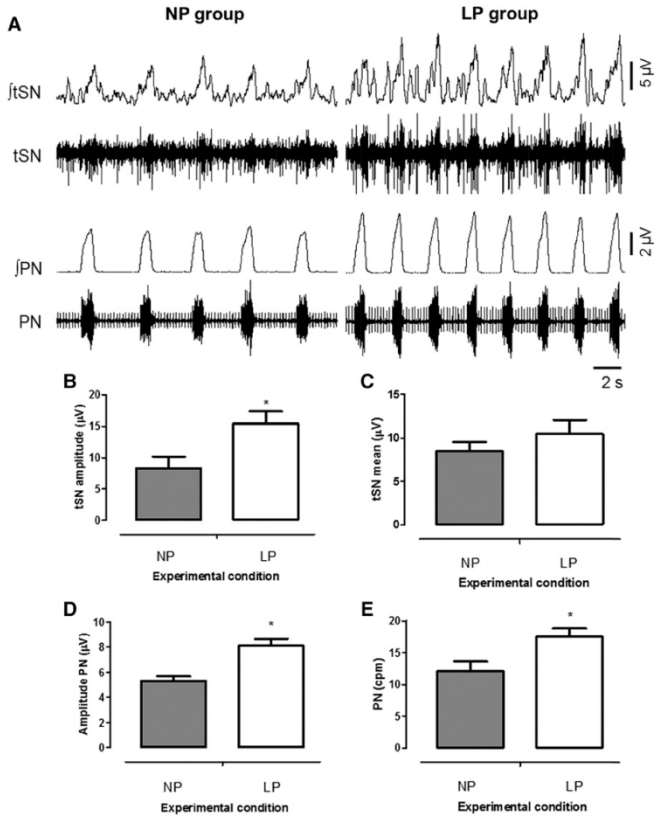

FIGURE 3 Representative tracings showing raw and integrated PN and tSN activities (A), average of baseline tSN amplitude (B), tSN mean (C), PN amplitude (D), and PN mean (E) for 30-d-old male rat pups of dams fed an NP or an LP diet during pregnancy and lactation. Values are means ± SEMs, $n = 6$–8. *Different from NP, $P < 0.05$ (unpaired Student's t test). cpm, cycles per minute; LP, offspring of experimental rat dams fed a low-protein diet (8% protein); NP, offspring of control rat dams fed a normoproteic diet (17% protein); PN, phrenic nerve; tSN, thoracic sympathetic nerve; ∫, integrated activity.

ventilation, as evidenced by greater respiratory frequency in vivo and higher phrenic burst frequency and amplitude in situ; 2) showed normal arterial pressure but augmented sympathetic activity at rest, as indicated by enhanced LF modulation of arterial pressure in unanesthetized rats and elevated levels of thoracic sympathetic activity in in situ preparations; and 3) amplified sympatho-excitatory and tachypnoeic responses to peripheral chemoreceptor stimulation combined with enhanced HIF-1α expression in the carotid bodies, suggesting a sensitization of the carotid peripheral chemoreceptors. Altogether, our data bring new insights into the etiologic mechanisms underlying the development of arterial hypertension in protein-restricted rats, highlighting a critical role of the sympathetic nervous system and peripheral chemoreceptors.

Although a relation between maternal protein restriction during pregnancy and lactation and the development of arterial hypertension in offspring during adult life has been previously described (6, 7, 29, 30), its underlying mechanisms are poorly understood. We recently reported that rat offspring subjected to protein undernutrition during pregnancy and lactation exhibited reduced ponderal gain and higher baseline arterial pressure at 90 d but not at 30 d of age. Despite the lack of augmented baseline arterial pressure, 30-d-old rats subjected to a perinatal LP diet exhibited an increased baseline respiratory frequency and enhanced respiratory responses to hypoxia and hypercapnia, suggesting that changes in the mechanisms controlling respiratory motor activity and/or increased oxygen/carbon dioxide chemosensitivity occur before the development of hypertension (7). Accordingly, in the present study, juvenile offspring from protein-restricted dams showed increased Rf in both the conscious state and in the in situ preparation. It was clearly shown that protein-restricted rats exhibited higher PN burst frequency and amplitude, indicating an increased central inspiratory activity at rest. Therefore, our data strongly support the notion that an LP diet during gestation and lactation elicits changes in the functioning of the central respiratory pattern and rhythm generator that precede the development of arterial hypertension.

Although arterial pressures were similar between the NP and LP groups at 30 d old, our data indicate that the sympathetic vasoconstrictor tonus of LP rats is enhanced. In unanesthetized rats, we verified that the variability at an LF range of SAP and pulse inteval, which are correlated with sympathetic drive to blood vessels and to the heart (23, 31), was significantly enhanced in the LP group. These observations suggest that maternal protein restriction can lead to augmented sympathetic modulation of the cardiovascular system in juvenile offspring. We also verified that in situ preparation of LP rats presented

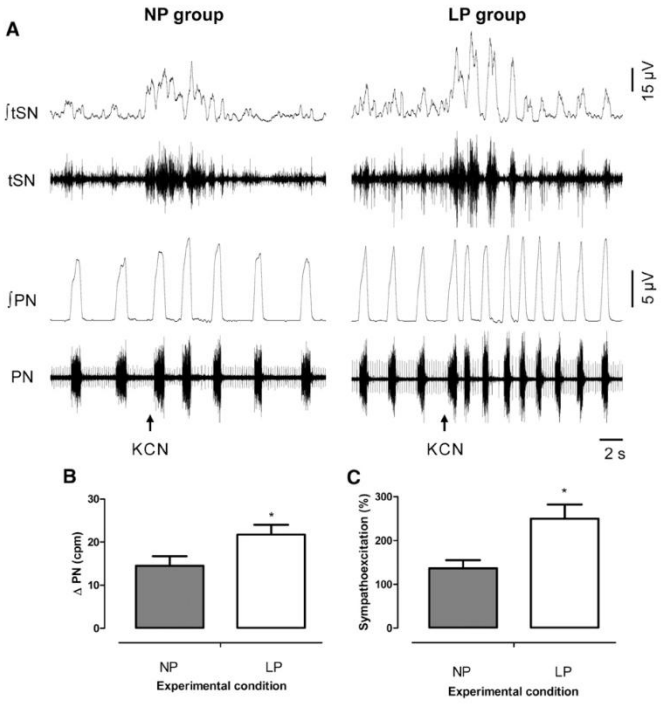

FIGURE 4 Representative tracings showing raw and integrated PN and tSN activities during peripheral chemoreflex activation (A) and averages of percentage tSN (B) and PN (C) during peripheral chemoreflex activation in 30-d-old male rat pups of dams fed an NP or an LP diet during pregnancy and lactation. Values are means ± SEMs, $n = 6$–8. *Different from NP, $P < 0.05$ (unpaired Student's t test). cpm, cycles per minute; KCN, potassium cyanide; LP, offspring of experimental rat dams fed a low-protein diet (8% protein); NP, offspring of control rat dams fed a normoproteic diet (17% protein); PN, phrenic nerve; tSN, thoracic sympathetic nerve; ∫, integrated activity.

increased levels of sympathetic nerve activity, supporting the notion that baseline sympathetic activity is indeed elevated in rats subjected to an LP diet during the perinatal period. This higher level of sympathetic activity observed before the onset of hypertension in LP rats suggests that dysfunction of the sympathetic nervous system, culminating in enhanced baseline sympathetic activity, contributes to the development of hypertension in these rats. These findings are in agreement with those observed in spontaneously hypertensive rats, which exhibit an elevated sympathetic activity in early life, just before the development of arterial hypertension (32, 33).

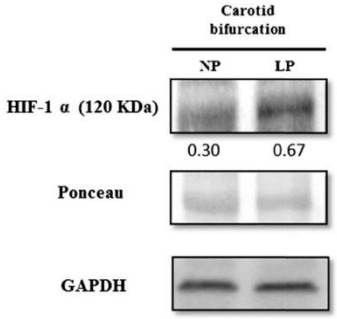

FIGURE 5 Western blot assay for expression of HIF-1α showing results presented to confirm equal loading of the protein samples. Relative densities of HIF-1α were normalized by the respective amounts of Ponceau S red in 30-d-old male rat pups of dams fed an NP or an LP diet during pregnancy and lactation. HIF-1α, hypoxia-inducible factor 1α; LP, offspring of experimental rat dams fed a low-protein diet (8% protein); NP, offspring of control rat dams fed a normoproteic diet (17% protein).

The role of the sympathetic nervous system in the generation of neurogenic hypertension has been convincingly supported with different experimental models (22, 32, 34–36). Because high levels of tSN activity in protein-restricted rats were associated with elevated Rf and increased PN burst frequency and amplitude, we hypothesize that the increased sympathetic drive of LP rats is linked to the increased baseline inspiratory activity. It is well established that the respiratory system markedly modulates sympathetic nerve discharge at rest (27, 34, 35, 37), introducing phasic bursts in sympathetic activity, mainly during the inspiratory/postinspiratory phases (27, 28, 35, 38). This respiratory modulation of sympathetic activity is consequent to synaptic interactions between respiratory and sympathetic neurons of the brainstem (37, 39–41). Presympathetic neurons that exhibit an inspiratory-modulated pattern of activity with increased frequency of discharge during inspiration have been identified within the rostral ventrolateral medulla (41).

Therefore, because baseline central inspiratory activity is enhanced in protein-restricted rats, we theorize that perinatal protein restriction increases the activity of inspiratory neurons that, in turn, send excitatory inputs to presympathetic neurons of the rostral ventrolateral medulla, thereby enhancing sympathetic activity primarily during inspiration (41). However, this hypothesis still requires further experimental verification.

In addition to baseline cardiorespiratory changes, the sympathetic and inspiratory reflex responses to peripheral chemoreceptor stimulation were also amplified in LP rats. These data are in agreement with our previous observation that the ventilatory responses to hypoxia are enhanced in unanesthetized LP rats at the same age (7), suggesting that peripheral chemoreflex is sensitized in rats subjected to perinatal protein restriction. In agreement with this hypothesis, we verified that the expression of HIF-1α is enhanced in the carotid bodies of these rats, revealing that this transcriptional factor, which is related to the response of cell to reduced oxygen and energy availabilities (42–44), may be involved in the adaptive responses elicited by protein restriction during the perinatal period (45) and mediates, at least in part, the cardiorespiratory changes observed in protein-restricted rats.

Recent experimental and clinical evidence indicates that dysfunction of carotid body chemoreceptors plays a critical role in the progression of cardiorespiratory morbidities associated with baseline sympathetic overactivity, including sleep disorders, congestive heart failure, chronic pulmonary obstructive disease, and hypertension (9, 12, 13, 27, 46–48). In agreement with this notion, we hypothesize that the enhanced baseline sympathetic and inspiratory motor activities of juvenile offspring from dams subjected to protein restriction during pregnancy and lactation, before the development of hypertension, are dependent, at least in part, on the sensitization of the peripheral chemoreflex. These represent new insights into the mechanisms underlying the arterial blood pressure control of rats that have undergone perinatal protein restriction.

In conclusion, the present study suggests that juvenile offspring from protein-restricted dams exhibit enhanced baseline sympathetic and inspiratory motor activities combined with amplified ventilatory and autonomic responses to peripheral chemoreflex activation before the establishment of hypertension. These changes are apparently associated with a high HIF-1α concentration in carotid body peripheral chemoreceptors. These findings can aid in understanding why blood pressure increases in individuals subjected to protein undernutrition during a critical period of life.

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JLdBA, CGL, DBZ, and JHC-S designed the research; JLdBA, VON, MPCN, DBZ, and JHC-S conducted the research; JLdBA, VON, MPCN, AML, CC, DSAC, EC, AGW, CGL, DBZ, and JHC-S analyzed the data and performed the statistical analysis; and JLdBA, DBZ, and JHC-S had primary responsibility for the final content. All authors read and approved the final manuscript.

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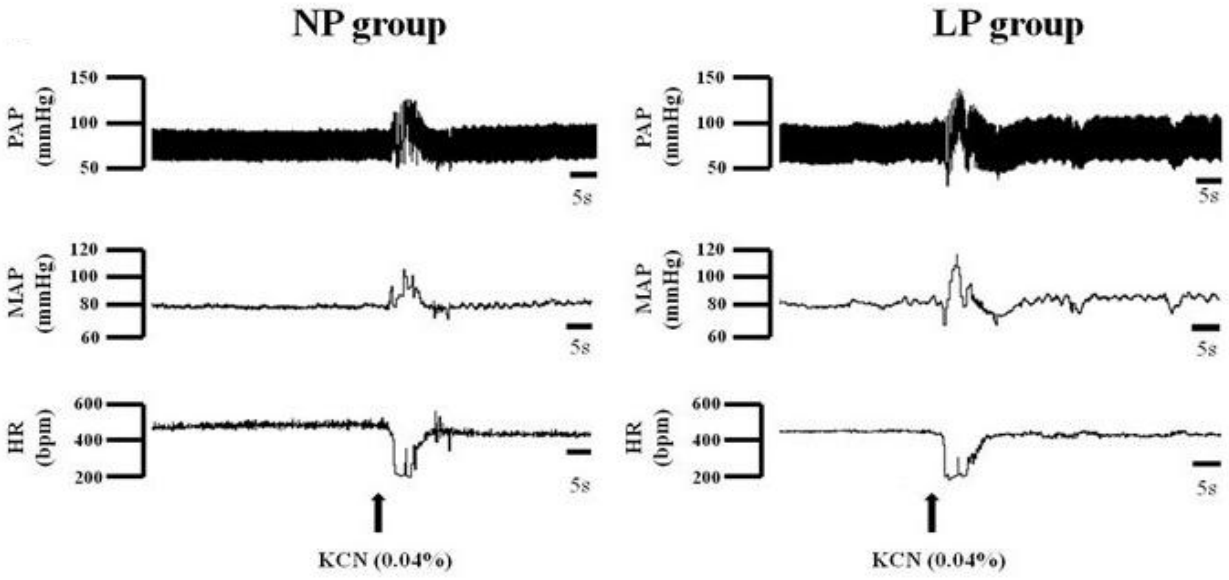
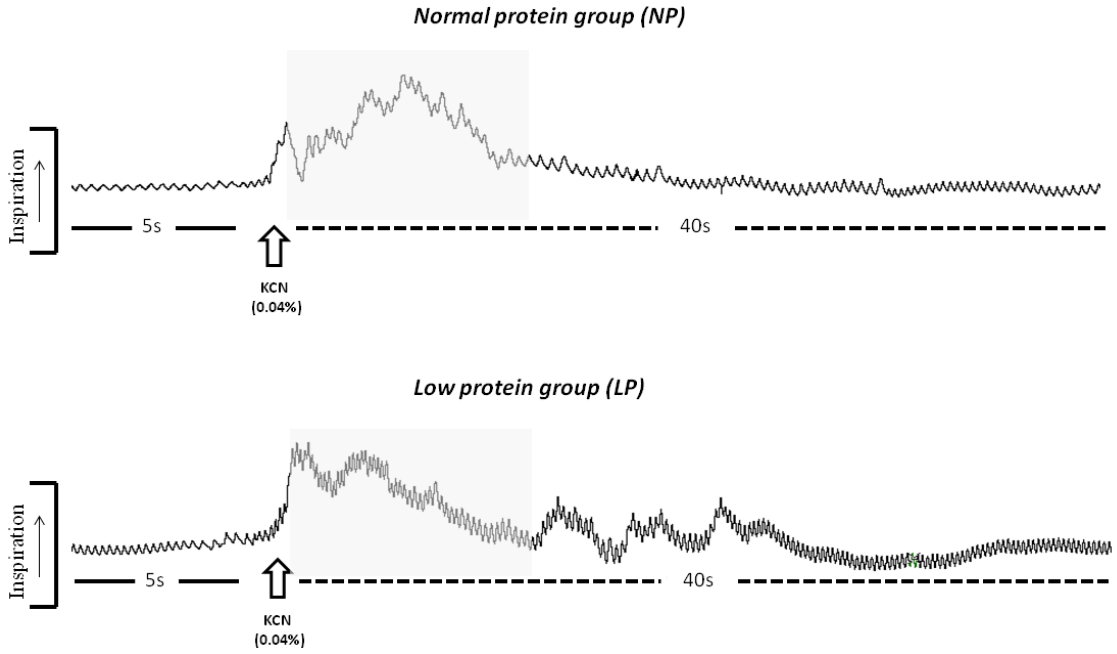


Supplemental Figure 1. Representative tracing of pulsatile arterial pressure (PAP),mean arterial pressure (MAP) and heart rate (HR) at rest and during peripheral chemoreflex activation (KCN, 0.04%) at 30-day old rats from mothers submitted to a normoproteic diet (NP, 17 % of protein) or low protein diet (LP, 8 % of protein) during pregnancy and lactation.



Supplemental Figure 2. Representative tracing of ventilation before and after peripheral chemoreflex activation (KCN, 0.04%) at 30-day old rats from mothers submitted to a normoproteic diet (NP, 17 % of protein) or low protein diet (LP, 8 % of protein) during pregnancy and lactation.

ARTIGO 03 – ARTIGO A SER SUBMETIDO AO JOURNAL OF RESPIRATORY, PHYSIOLOGY AND NEUROBIOLOGY

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ARTIGO 03: Short-term effects of perinatal protein restriction on CO_2 chemosensitivity and expression of oxidative-glutamatergic genes in medulla in male rat offspring

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Conflict of interest statement

The authors declare no competing financial interests.

Abstract

Maternal protein restriction is a predisposing factor to the development of adult arterial hypertension by unknown mechanisms. Here, we investigated whether low-protein diet during gestation and lactation enhances the respiratory and sympathetic responses to central chemoreceptors onset before hypertension. Male Wistar rats were subjected to control (NP, 17% of protein) or low-protein (LP, 8% of protein) diet during gestation and lactation. At 30-day old, recordings of phrenic (PN) and thoracic sympathetic nerve activities (tSN) performed in the *in situ* preparations of NP (n=6) and LP rats (n=8), revealed that LP rats presented ($P<0.05$) larger PN burst frequency-amplitude during baseline and hypercapnia condition in comparison to NP group. In addition, genes expression of superoxide dismutase (SOD2), catalase, glutathione peroxidase (GPX), glutamatergic receptor AMPA1 (Gria1), glutamatergic receptor NMDA 1 (Grin1) and glutamatergic receptor metabotropic 1 (Grm1) were performed in medulla of NP and LP group and reveled that all genes expression are not changed under both conditions. The present data show that perinatal LP diet alters CO2 respiratory chemosensitivity at early ages, which was not linked to changes in the antioxidant system and glutamatergic receptors gene expression in medulla.

Key words: maternal protein restriction, sympathetic activity, phrenic activity, CO_2 chemoreception, genes expression.

Introduction

Arterial hypertension is a complex trait determined by both genetic and environmental factors considered as the most important risk factors of cardiovascular disease (Choi *et al.*, 2015). However, the identification of the predisposing factors has been difficult due the multifactorial nature of the hypertension.

Interestingly, experimental and epidemiological studies have showed that perinatal malnutrition can contribute to the development of arterial hypertension (AH) in adult offspring (Roseboom *et al.*, 1999; Mcmullen e Langley-Evans, 2005; De Brito Alves, Nogueira*, et al.*, 2014b; Barros *et al.*, 2015). In this way, we recently showed that rats subjected to protein restriction during gestation and lactation exhibit AH in adulthood (De Brito Alves, Nogueira*, et al.*, 2014b; Barros *et al.*, 2015).

On the other hand, before the onset of hypertension, protein-restricted rats exhibited enhanced sympathetic and respiratory activities combined with high peripheral chemoreceptors sensitivity (De Brito Alves *et al.*, 2015). Thus, we hypothesized that short-term alteration in sympathetic-respiratory-quimiossensitivity network would predispose to the development of arterial hypertension in protein-restricted rats.

Beyond of peripheral arterial chemoreceptors, the central chemoreceptors also are involved in the control of ventilation and sympathetic activity. Previous studies demonstrated that the CO_2 provides tonic drive in respiratory and pre-sympathetic neurons located in medulla (Spyer e Gourine, 2009; Molkov *et al.*, 2011).

The medullary region contains critical site important for the generation of sympathetic and respiratory activities (Feldman *et al.*, 2003; Guyenet, 2006). In this region, CO_2-activated glutamatergic chemosensitive neurons contribute in various ways to the generation of the respiratory and sympathetic pattern (Costa-Silva *et al.*, 2010; Dean e Nattie, 2010; Guyenet *et al.*, 2010).

Previous studies have shown that oxidative stress may affect the ventilatory control and central chemosensitity (Zakynthinos *et al.*, 2007). Mechanistically, it was demonstrated that some CO_2 chemosensitivity neurons are excited by the reactive oxygen species, probably due a acidification in medullary neurons (Zakynthinos *et al.*, 2007), which may contribute eventually for respiratory dysfunction and chemosensory (Dean *et al.*, 2004; Mulkey *et al.*, 2004).

Interestingly, we demonstrated *in vivo* that rats subjected to perinatal protein undernutrition present amplified ventilatory responses to hypercapnia (gaseous mixture with 7% CO_2) (De Brito Alves, Nogueira*, et al.*, 2014b). However, there is no direct evidence

demonstrating that sympathetic-respiratory response to the CO_2 are elevated in this model and if the sympathetic-respiratory overactivity it is combined with higher expression of oxidative and glutamatergic genes expression in the medulla.

In this context, in the present study we hypothesized that juvenile rats from mothers subjected to protein restriction during pregnancy and lactation would exhibit amplified respiratory and sympathetic responses to CO_2 chemoreceptors activation combined with higher expression of glutamatergic genes and oxidative stress in the medulla.

Methods

The experimental protocol was approved by the Ethical Committee of the Biological Sciences Centre (protocol 044454/2010-94), Federal University of Pernambuco and by Animal Experimentation Ethics Committee of the School of Dentistry of Araraquara at the São Paulo State University (protocol 21/2012), Brazil. All efforts were made to minimize animal discomfort and the number of animals used; in addition, we followed the Guidelines for the Care and Use of Laboratory Animals.

Animals and experimental groups

Virgin female albino Wistar rats (*Rattus novergicus*) were obtained from the Academic Centre of Vitoria de Santo Antão (CAV), Federal University of Pernambuco, Brazil. Animals were maintained at room temperature of 22 ± 1^{o}C with controlled light–dark cycle (dark 18:00–06:00 hours). Standard laboratory chow (52% carbohydrate, 21% protein, and 4% lipids - Labina®, Purina Agriband, São Paulo, Brazil) and water were given *ad libitum* up to the 3-month, when rats were mated (2 females for 1 male). The day on which spermatozoa were identified in vaginal smear was considered as the conception and the pregnant rats were transferred to individual cages. Two experimental groups were designed according to the diet manipulation: mothers fed with 17% casein diet (normal-protein group, NP, n=6) and mothers fed with 8% casein diet (low-protein group, LP, n=6) and water *ad libitum*. Both diets were isoenergetic (**Table 1**) and were offered during pregnancy and lactation. During the suckling period, the offspring were maintained as litters of eight pups randomly. At weaning, three or four male offspring of each litter were randomly housed in collective cages and received a standard diet *ad libitum*. Diets were elaborated at the Laboratory of Experimental Nutrition-CAV, Federal University of Pernambuco, according to the American Institute of Nutrition – AIN-93 (Reeves *et al.*, 1993). The casein was previously analysed and showed 85% of purity (85g of protein for each 100g of casein). The

experimental groups were formed with two or three rats from each mother and all experiments were performed in 30-day old juvenile rats.

***In situ* working heart–brainstem preparation (WHBP)**

The juvenile rats (Np, n=6 and Lp, n=7) were deeply anesthetized with halothane (Astra Zeneca, Cotia, SP, Brazil) such that the withdrawal responses to noxious pinching of the tail and paw were absent. The animals were then transected caudal to the diaphragm and submerged in a cooled Ringer solution (in mM: 125 NaCl, 24 $NaHCO_3$, 3 KCl, 2.5 $CaCl_2$, 1.25 $MgSO_4$, 1.25 KH_2PO_4 and 10 dextrose). They were decerebrated at the precollicular level to make insentient, skinned, and had descending aorta isolated. Preparations were then transferred to a recording chamber, the descending aorta was cannulated and perfused retrogradely with modified Ringer solution containing lactate (2 mM), an oncotic agent (1.25% polyethylene glycol, Sigma, St Louis, MO) and a neuromuscular blocker (vecuronium bromide, 3–4 µg/ml, Cristalia, Itapira, SP, Brazil), using a roller pumps (Watson-Marlow 502s, Falmouth, Cornwall, UK) via a double-lumen cannula. The perfusion pressure was maintained in the range of 50 –70 mmHg by adjusting the rate flow between 21 and 25 ml/min and by adding vasopressin to the perfusate (6–12 nM, Sigma) as previously described (Zoccal *et al.*, 2008). The perfusate was gassed continuously with 5% CO_2 – 95% O_2, warmed to 31-32 ºC, and filtered using a nylon mesh (pore size: 25 µm, Millipore, Billirica, MA).

Electrical activities from all nerves were obtained using glass suction bipolar electrodes held in a micromanipulator (Narishige, Tokyo, Japan). Left phrenic nerve (PN) discharges were recorded from its central end and its rhythmic ramping activity gave a continuous physiological index of preparation viability. Thoracic sympathetic nerve (tSN) activity was recorded from thoracic sympathetic chain, at the level of T10 –T12. All signals were amplified, band-pass filtered (0.05–5 kHz), and acquired in an A/D converter (CED 1401, Cambridge Electronic Design, Cambridge, UK) to a computer using Spike2 software (version 7, CED).

All analyses were performed off-line in rectified and integrated (50 ms) signals using Spike 2 software with custom-written scripts. Before analyses, PN and tSN recordings were subtracted from the electrical noise obtained after the death of WHBP (induced by pump off). For baseline measurements, PN activity was assessed by its frequency (cycles per minute, cpm), amplitude (µV), burst duration (inspiratory time, s) and burst interval (expiratory time, s). tSN activity was assessed by its mean activity (µV) and by the amplitude of inspiratory-

related bursts (µV). The later was calculated by the value difference between the maximal and the lowest activity observed during inspiratory and post-inspiratory phases.

Hypercapinc Stimuli

Using a gas mixer device (GF3/MP gas mixing flowmeter; Cameron Instrument, Port Aransas, TX), the proportion of the gases in the perfusate was altered to raise or lower CO_2. For hypercapnic stimuli, the concentrations were 7% CO_2-93% O_2 and 10% CO_2-90% O_2, whereas for hypocapnic stimuli, the concentrations were 3% CO_2-97% O_2 and 1% CO_2-99% O_2. The time duration of exposure for each stimulus was at 3-4 min.

Medulla preparation

Rat medulla were collected using as reference the calamus scriptorium (CS), wherein we used approximately 2 mm rostral and 2 mm caudal from CS, according to the stereotaxic atlas.

RNA extraction, reverse transcription and quantitative PCR (qPCR).

Separate groups of Np (n = 5) and Lp (n = 5) rats that were not subjected to any surgical procedure were killed by decapitation and their medulla were collected. Total RNA was extracted from medulla tissues with Tripure reagent (Roche, Meylan, France) according to the manufacturer's instructions. Briefly, 10µL of Trizol were added per mg of tissue and the resulting suspension was homogenized using a Precellys Lysing kit (Bertin, Montigny-le-Bretonneux, France) according to the manufacturer's instructions. After grinding, 1/4 volume of chloroform was added, the preparation vortexed 3 x 15 s, incubated at room temperature for 5 min and centrifuged for 15 min at 15,000 g at 4°C. RNA was precipitated by addition of 1/2 volume of isopropanol (Carlo Erba, Val-de-Reuil, France) and centrifugation (15 min at 15,000g at 4°C). RNA-containing pellets were washed sequentially with 70% and 95% ethanol (Carlo Erba), dried, and dissolved in 100 µl RNase-free water.

Reverse transcription was performed using an RT-TAKARA kit (Primescript TM, Dalian, Japan) using 1µg of RNA as template and following the manufacturer's instructions. Briefly, samples were heated for 10 min at 65 °C. 4µL PrimeScript Buffer 5x, 1µl oligodT (50 µM), 4µl random hexamers and 1µl of PrimeScript RT Enzyme Mix, were sequentially added, followed by a 15 min incubation at 37 °C and 15 s at 85 °C. RNA was removed by incubation with 1µL of RNase H for 20 minutes at 37 °C. Reverse transcription reactions were brought to 200 µl final volume by adding RNase free water, and stored at -20°C. Real-time quantitative

PCR amplification (qPCR) was performed using a Rotor-Gene Real-Time PCR System (Labgene Scientific Instruments, Archamps, France). Sequences of primers used in this study are reported in supplementary Table 2.

Reactions were incubated at 95°C for 10 min, followed by 40 cycles of denaturation (95°C, 10 s), annealing (58-62 °C depending on the primer sets, 30 s) and elongation (72 °C, 30 s). mRNA expression levels are represented as arbitrary units (A.U.) derived from a standard calibration curve derived from a reference sample. A PCR for each sample was carried out in duplicate for all cDNAs and for the ribosomal protein L19 control. As a further control, qPCR amplicons were analyzed by agarose gel.

Statistical Analysis

Data homogeneity was checked by the Bartlett's method and statistical differences between the groups and within the groups were assessed by unpaired and paired Student's t–test, respectively. Significance level was fixed at $P<0.05$. Data were expressed as mean ± standard error (SE) and statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA).

Results

Figure 1 summarizes the recordings of phrenic (PN) and thoracic sympathetic activities (tSN) during baseline and hypercapnia condition of representative 30-day old rats from the NP (n=6) and LP (n=8) groups. Average tSN levels were not statistically different between groups during normoxia and hypercapnic stimuli ($P>0.05$; **Fig 2a e b**). In baseline condition, LP rats exhibited larger PN burst frequency ($P<0.05$, **Fig. 2c**) and amplitude ($P<0.05$; **Fig.2d**). During hypercapnia condition, LP rats exhibited enhanced PN frequency and amplitude at 7% and 10% CO2 ($P<0.05$; **Fig 2c and d**) in comparison to Np group.

mRNA expression of the genes involved in oxidative stress and glutamatergic genes were analyzed in the medulla of protein-restricted rats. The SOD 2 (Fig. 3b), catalase (Fig. 3c) and GPX (Fig. 3d) mRNA expression were similar between Np and Lp groups. In parallel, mRNA expression for Grin1 (Fig. 3e), Gria1 (Fig. 3f) and Grm1 (Fig. 3g) were also similar between groups.

Discussion

In the present study, we investigated the short-term effects of protein restriction during pregnancy and lactation on CO_2-chemosensitivity and medullary gene expression in male

offspring. The main findings of this study showed that protein restriction during perinatal development produced in 30-d-old rats amplified tachypnoeic responses to CO_2 stimuli, but no change in sympatho-excitatory response. In addition, mRNA expression for SOD2, catalase, GPX, Grin1, Gria1 and Grm1 in the medulla was not changed between Np and Lp group.

Interestingly, we demonstrated *in vivo* that the ventilatory response to CO_2 was enhanced in rats subjected to perinatal protein restriction (De Brito Alves, Nogueira, *et al.*, 2014b), but when we performed hypercapnic stimuli, the pressor response to CO_2 it was similar between Np and Lp groups (unpublished data). Similarly, our findings in *in situ* preparation showed that the frequency and amplitude of PN bursts was enhanced in response to CO_2, indicating an increased central respiratory chemosensitivity.

Our group, studying the involvement of peripheral chemoreceptors in the ventilatory and sympathetic activities of protein-restricted rats, observed an augmented respiratory and sympatho-excitatory response during peripheral chemoreflex activation combined with enhanced HIF-1a expression in the carotid bodies, suggesting a higher sensitization of the carotid peripheral chemoreceptors (De Brito Alves *et al.*, 2015).

On the other hand, our data showed that the sympathetic response to CO_2 stimuli was not changed between Np and Lp groups. Therefore, we speculated that the sympathetic hyperactivity verified in protein-restricted rats are strongly dependent on carotid body inputs.

It has been show that abnormalities in gene expression of the chemosensory transducers in the brainstem could be related in the on respiratory ventilatory response to high CO_2 (Tan *et al.*, 2010; Huckstepp e Dale, 2011). Previously, has been verified that excessive production and accumulation of reactive oxygen species (ROS) in the brainstem might affect CO_2 chemoreceptors and ventilation, leading to a large stimulation of firing rate of CO2-sensitive neurons (Dean, 2010; Iturriaga *et al.*, 2015).

Previous reports have shown that glutamatergic inputs into NTS and RVLM exhibit a key role in the phrenic and sympathetic coupling and CO_2 chemosensitivity in rats submitted to chronic intermittent hypoxia (Accorsi-Mendonca *et al.*, 2009; Costa-Silva *et al.*, 2010; 2012; Moraes, Bonagamba, *et al.*, 2014). In this way, studies performed in offspring subjected to protein-restricted post-natal demonstrated change in gene expression involved in aspartate and glutamate metabolism, which play an important role in the excitatory neurotransmission of the central nervous system (Rodrigues *et al.*, 2012). Analyzing glutamatergic genes expression in medulla from protein-restricted rats at 30-d-old, we did not observe alteration in mRNA expression for Grin1A, Gria1A and Grm1 between Np and Lp rats.

It was showed that noradrenaline and serotonin are important neuromodulators involved in the hypercapnic ventilator response (Zhang *et al.*, 2011; Toward *et al.*, 2013). In this way, it was shown that depletion of 5-HT neurons decrease the respiratory response to CO_2 by 32% (Da Silva *et al.*, 2011). Conversely, selective stimulation of serotoninergic raphe obscurus neurons enhanced ventilation and ventilator response to high CO_2 in mice (Calcagno *et al.*, 2007; Depuy *et al.*, 2011). The involvement of noradrenergic and serotoninergic gene expression in central nervous system of protein-restricted offspring are worthy of future investigation.

In conclusion, the present data showed that short-term effects induced by a protein-restricted diet during the perinatal period included increased respiratory rhythm and CO_2 chemosensitivity, which was not linked to changes in the glutamatergic receptors and antioxidant enzymes gene expression. These findings provide insights into the effects of maternal protein restriction and suggest that increased CO_2 chemosensitivity is one possible risk factor for the development of arterial hypertension in protein-restricted rats.

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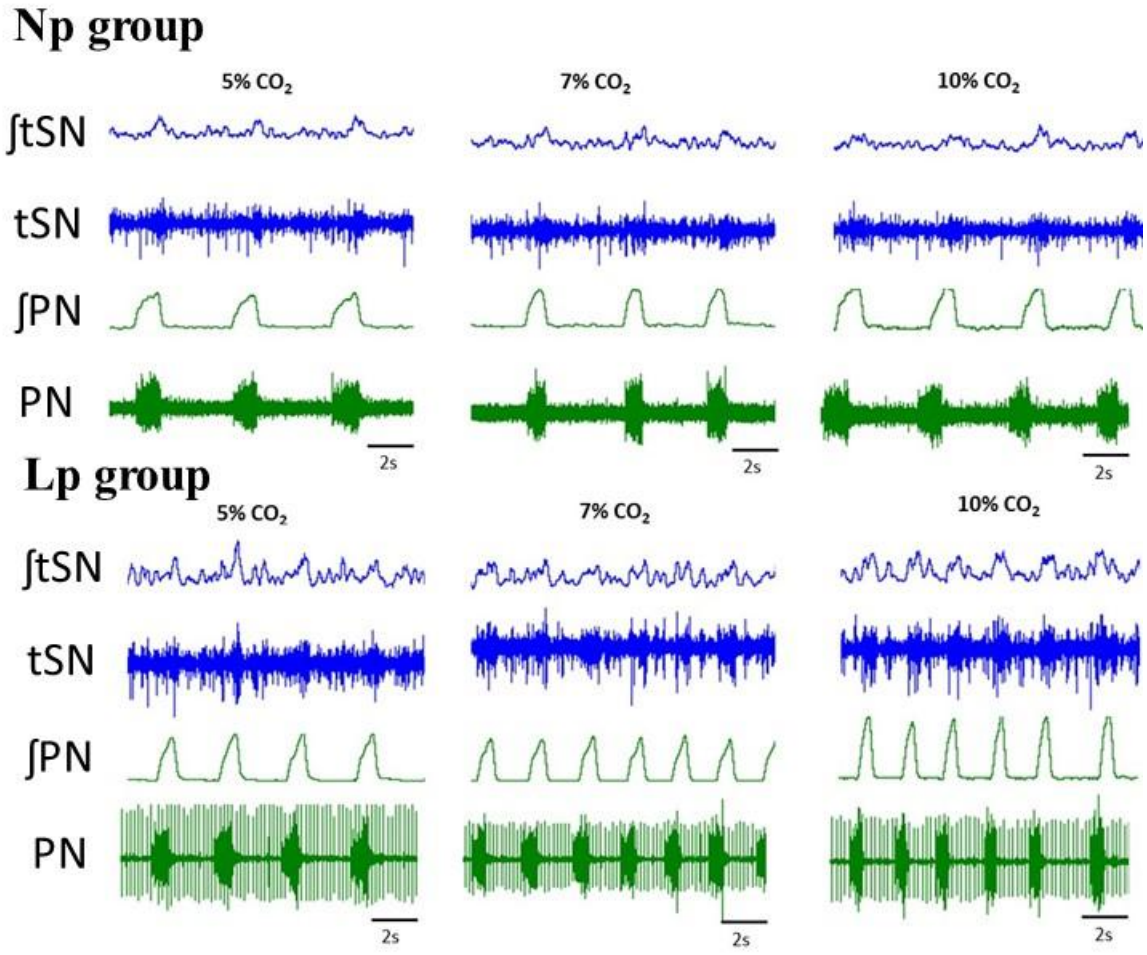

FIGURE 1. Representative tracings showing raw and integrated PN and tSN activities during baseline condition (5% CO_2) and hypercapnia (7% CO_2 and 10% CO_2) in 30-d-old male rat pups of dams fed an NP or an LP diet during pregnancy and lactation.

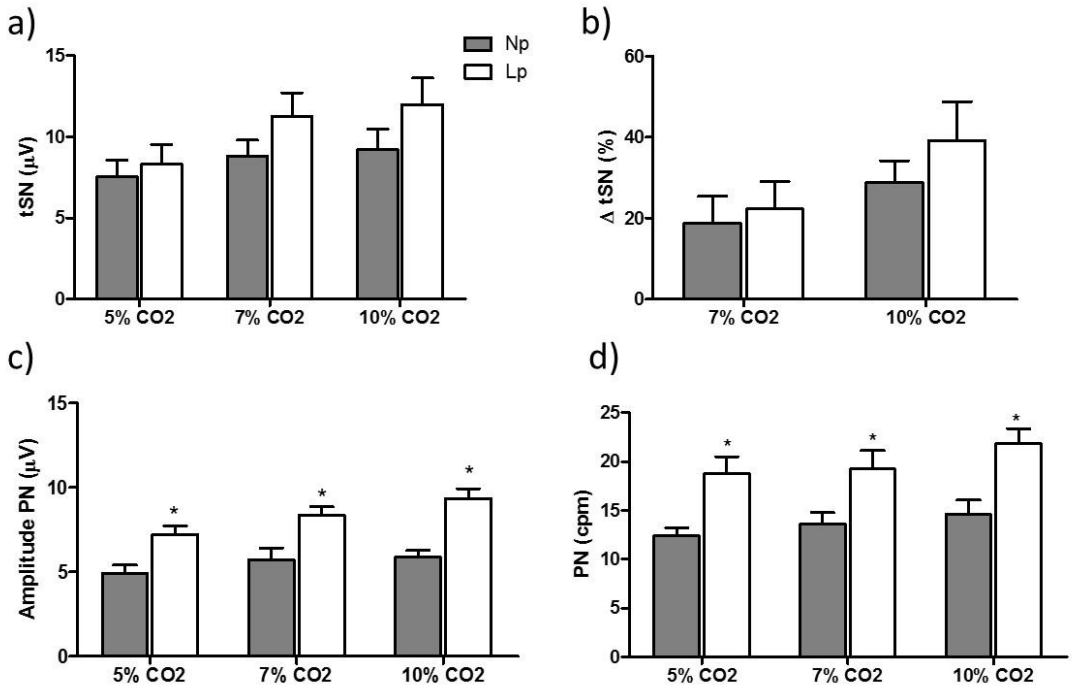

FIGURE 2. Averages baseline and during hypercapnia of tSN mean (a), Δ% tSN (b), PN amplitude (c) and PN mean (d) for 30-d-old male rat pups of dams fed an NP or an LP diet during pregnancy and lactation. Values are means 6 SEMs, n = 6–8. *Different from NP, $P < 0.05$ (unpaired Student's t test). cpm, cycles per minute; LP, offspring of experimental rat dams fed a low-protein diet (8% protein); NP, offspring of control rat dams fed a normoproteic diet (17% protein); PN, phrenic nerve; tSN, thoracic sympathetic nerve, integrated activity.

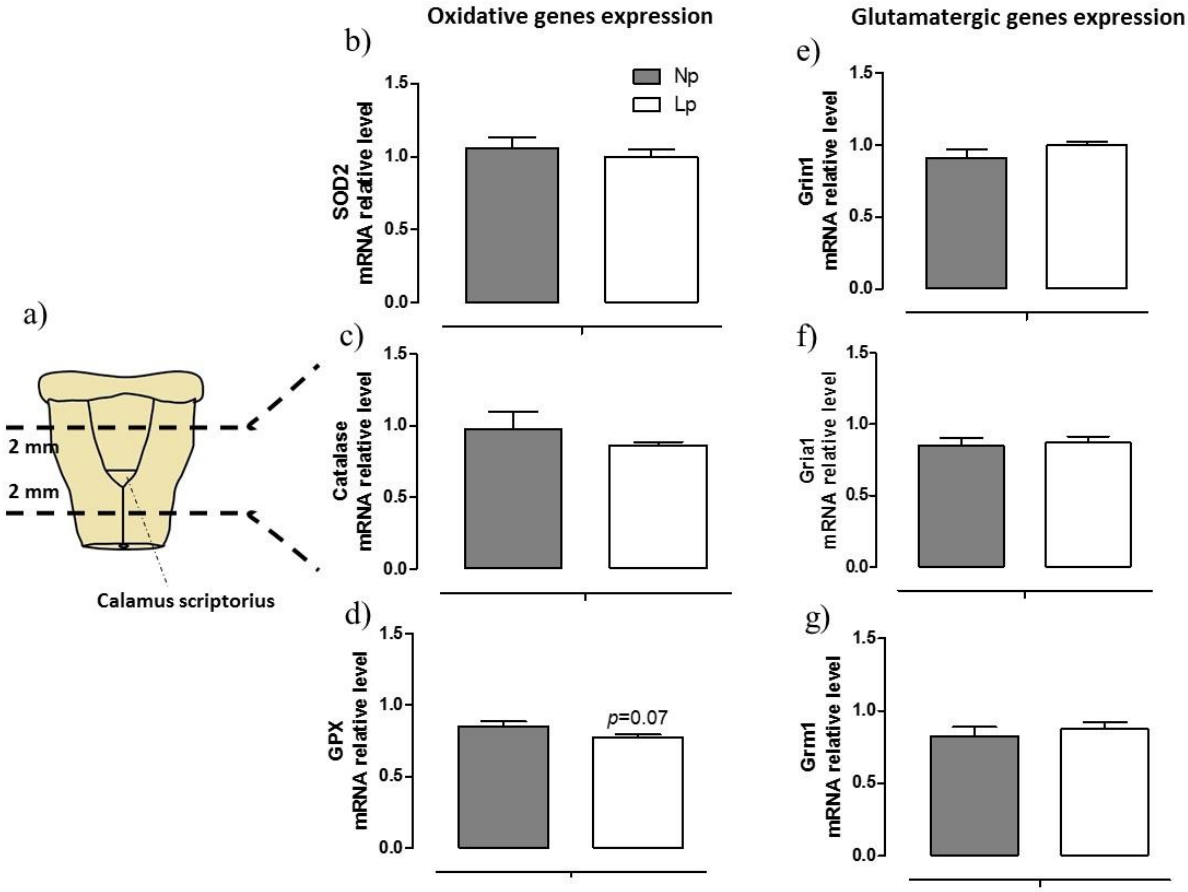

FIGURE 3. Evaluation of mRNA of SOD2 (b), catalase (c), GPX (d), Grin 1 (e), Gria 1 (f) and Grm 1 (g) in medulla**.** Gray bars represent Np group (17% protein, n=5) and white bars Lp group (8% protein, n=5). All pups were fed a standard chow diet at weaning. Values are mean±SEM (*P<0.05, Student's t-test).

Supplemental Table 1. Nutritional Composition of the experimental diets (g/100g diet)

Nutrient	Normal protein (17% protein)	Low protein (8% protein)
Casein (85%)*	20	9.41
Dextrin cornstarch	13	13.2
Cellulose	5	5
Sucrose	10	10
Cornstarch	39.74	50.34
Soybean oil	7	7
Choline	0.25	0,25
Methionine	0.3	0.3
Vitamin mix	1	1
Mineral mix	3.5	3.5
Energy density (kJ/g)	16.26	16.26

* The casein used in preparation of diet was 85% purity.

Table 2. Primers sequence used to perform qRT-PCR

Gene	Foward/Reverse	Tm	Sequence 5'-3'	Amplicon size, bp
Catalase	F	60°C	CATCGGCACATGAATGGCTA	281pb
	R		ACCTTGGTCAGGTCAAATGG	
GPX	F		TGAAGAGAGGCATGTTGGAG	
	R		GCTTCCCTTGCAACCAGTTC	189 pb
SOD 2	F	60°C	CATAAGGGTAGGGCAGCTTG	
	R		CCATTGAACTTCAGTGCAGG	138pb
Grin 1	F	60°C	TACAACTGGAACCACATCATCC	
	R		TGTCATAGGACAGTTGGTCGAG	145 pb
Gria 1	F	60°C	TGTTTGCCTACATTGGAGTGAG	
	R		GAACCACAGGCTGTTGAATATG	149 pb
Grm 1	F	60°C	CTATCATAGCCATCGCCTTTTC	
	R		GAGGAAAATACCAGCCAGAATG	143 pb
RPL19	F	58°C	CTGAAGGTCAAAGGGAATGTG	
	R		GGACAGAGTCTTGATGATCTC	195pb

ARTIGO 04 – ARTIGO A SER SUBMETIDO AO NUTRITIONAL NEUROSCIENCE JOURNAL

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ARTIGO 04: Oxidative status into ventral surface of the medulla can be important to development of arterial hypertension in protein-restricted rats

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Abstract

Background and objectives: Maternal protein restriction during pregnancy and lactation is a predisposing factor to development of sympathetic overactivation and arterial hypertension in adult offspring. However, the underlying mechanism are poorly understood. The aims of the study were to evaluate the effects of a low-protein (Lp) diet during pregnancy and lactation on oxidative and glutamatergic balance in the medulla of adult offspring. **Methods and results**: Wistar rat dams were fed a control [normal-protein (NP); 17% protein] or an LP (8% protein) diet during pregnancy and lactation, and their male offspring were studied at 90 d of age. Direct measurements of baseline arterial blood pressure (ABP) and heart rate (HR) were recorded in awake offspring. In addition, genes expression of SOD1, SOD2, catalase, Gria1, Grin1 and Grm1 were evaluated in medulla though Rt-PCR assay and MDA levels, Catalase and SOD activity were analyzed in ventral and dorsal medulla. Lp rats exhibited higher levels in arterial blood pressure. The SOD2 gene was down regulated (approximately 20%) in medulla of Lp rats. In addition, we observed higher MDA levels and decrease in the SOD (approximately 45%) and catalase (approximately 50%) in ventral medulla. **Discussion**: Our data showed that offspring of dams subjected to protein restriction during pregnancy and lactation exhibit high arterial blood pressure associated with imbalance in oxidative status in medulla ventral. This data can help in understanding why hypertension increase in rats exposed to protein restriction during pregnancy and lactatation.

Keywords: Maternal protein restriction, hypertension, medulla, oxidative stress.

Introduction

Adverse events experienced in utero or during the perinatal life (gestation, lactation and first infancy) has been propose as predisposing factor to the development of arterial hypertension and metabolic diseases in later life (Mcmullen e Langley-Evans, 2005; Costa-Silva *et al.*, 2009; De Brito Alves, Nogueira, *et al.*, 2014b; M.A.V. Barros, 2014). Although a relationship between maternal protein restriction during pregnancy and lactation and the development of arterial hypertension in offspring at adult life has been previously describe, its underlying mechanisms are poorly understood.

Recently, our laboratory reported that rats subjected to maternal protein restriction during pregnancy and lactation exhibit enhanced arterial blood pressure associated with sympathetic and respiratory overactivity (De Brito Alves, Nogueira, *et al.*, 2014b; Barros *et al.*, 2015; De Brito Alves *et al.*, 2015). However, they possible mechanisms that predispose hypertension and augmentation of sympathetic-respiratory activities are yet poorly understood.

The role of the sympathetic nervous system in the generation of neurogenic hypertension has been convincingly supported with different experimental models (Zoccal *et al.*, 2008; De Brito Alves *et al.*, 2015). Presympathetic neurons that exhibit an inspiratory-modulatory pattern activity have been identified within the rostral ventrolateral medulla (RVLM) and their higher activity is associated with sympathetic overactivity and development of arterial hypertension.

Interestingly, reactive oxygen species (ROS) into RVLM have a potential role in modulation of sympathetic activity and blood pressure (Nishihara, Hirooka, Kishi, *et al.*, 2012; Braga, 2013; Sousa *et al.*, 2015). For example, spontaneously hypertensive rats (SHR) exhibit increased lipid peroxidation measured by malondialdehyde (MDA) levels in the RVLM (Nishihara, Hirooka, Matsukawa, *et al.*, 2012). On the other hand, studies have demonstrated that antioxidant enzymatic complex evidenced through of superoxide dismutase (SOD) and catalase (CAT) activity are reduced into the RVLM of hypertensive rats (Chan *et al.*, 2006; Hirooka *et al.*, 2010), suggesting that oxidative stress can contribute to higher sympathetic activity and arterial hypertension.

Despite these findings, the involvement of oxidative stress in the ventral or dorsal medulla of protein-restricted rats remains undetermined. In this context, in the present study, we hypothesized that adult rats from dams subjected to protein restriction during pregnancy and lactation could exhibit an oxidative imbalance in the ventral medulla that contribute, in part, to the development of arterial hypertension in protein-restricted rats.

Methods

The experimental protocol was approved by the Ethical Committee of the Biological Sciences Centre (protocol 23076 019345/2013-81), Federal University of Pernambuco, Brazil. All efforts were made to minimize animal discomfort and the number of animals used; in addition, we followed the Guidelines for the Care and Use of Laboratory Animals.

Animals and experimental groups

Virgin female albino Wistar rats (Rattus novergicus) were obtained from the Academic Centre of Vitoria de Santo Antão (CAV), Federal University of Pernambuco, Brazil. Animals were maintained at room temperature of 22 ± 1°C with controlled light–dark cycle (dark 18:00–06:00 hours). Standard laboratory chow (52% carbohydrate, 21% protein, and 4% lipids - Labina, Purina Agriband, São Paulo, Brazil) and water were given ad libitum up to the 3-month, when rats were mated (2 females for 1 male). The day on which spermatozoa were identified in vaginal smear was considered as the conception and the pregnant rats were transferred to individual cages: mothers fed with 17% protein diet (n=5, normal protein group, Np) and mothers fed with 8% casein diet (low protein group, Lp, n=5) and water ad libitum. Both diets were isoenergetic (**Table 1**) and were offered during pregnancy and lactation. The low protein diet differed from the normal protein diet in the content of protein and carbohydrate. Diets were elaborated at the Laboratory of Experimental Nutrition-CAV, Federal University of Pernambuco, according to the American Institute of Nutrition – AIN-93 (Reeves *et al.*, 1993). The casein was previously analyzed and showed 85% of purity (85g of protein for each 100g of casein).

During the suckling period, the offspring were maintained as litters of eight pups randomly. At weaning, three or four male offspring of each litter were randomly housed in collective cages and received a standard diet and water ad libitum. The experimental groups were formed with one or two or rats from each mother. For expression genes analyze were used n=5 Np or Lp rats. In addition, for enzymatic assay cardiovascular evaluations *in vivo* we used n=7 of each group.

Cardiovascular evaluations *in vivo*

For confirm the effects of maternal protein restriction on arterial blood pressure of offspring, one day before of the experiments, Np (n=7), Lp (n=7) rats were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg), and the femoral artery was cannulated (PE-50

connected to PE- 10; Clay Adams, Parsippany, NJ, USA) as describe previously by de Brito Alves *et al* (De Brito Alves, Nogueira, *et al.*, 2014b) and Barros *et al*. (Barros *et al.*, 2015)

Briefly, the catheters were filled with heparinized saline (NaCl 0.9%), tunneled subcutaneously and exteriorized through the back of the neck. After surgery, the animals received injection of ketoprofen (5 mg/kg ip) and a period of 24 hours were allowed until rats fully recovered from the surgical and anaesthetic procedures. After the next day, rats were acclimatized during 1 hour and mean arterial pressure (MAP) and heart rate (HR) baseline were recorded for 50 min in unanesthetized freely-moving animals by connecting the arterial catheter to a pressure transducer. The signals were amplified (ML866/P, ADInstruments, Power Lab, Bella Vista, NSW, Australia). All data were analysed off-line using appropriate software (LabChart 7 Pro, ADInstruments).

Medulla preparation

Rat medulla were collected using as reference the calamus scriptorium (CS), wherein we used approximately 3 mm rostral and 2 mm caudal from CS, as previous described and in stereotaxic atlas. For gene expression, experiments were used medulla completely and for enzymatic assay, the medulla were sectioned in ventral and dorsal area.

RNA extraction, reverse transcription and quantitative PCR (qPCR).

Under normoxic conditions, separate groups of Np (n = 5) and Lp (n = 5) rats that were not subjected to any surgical procedure were killed by decapitation and their medulla were collected.

Total RNA was extracted from medulla tissues with Tripure reagent (Roche, Meylan, France) according to the manufacturer's instructions. Briefly, 10µL of Trizol were added per mg of tissue and the resulting suspension was homogenized using a Precellys Lysing kit (Bertin, Montigny-le-Bretonneux, France) according to the manufacturer's instructions. After grinding, 1/4 volume of chloroform was added, the preparation vortexed 3 x 15 s, incubated at room temperature for 5 min and centrifuged for 15 min at 15,000 g at 4°C. RNA was precipitated by addition of 1/2 volume of isopropanol (Carlo Erba, Val-de-Reuil, France) and centrifugation (15 min at 15,000g at 4°C). RNA-containing pellets were washed sequentially with 70% and 95% ethanol (Carlo Erba), dried, and dissolved in 100 µl RNase-free water.

Reverse transcription was performed using an RT-TAKARA kit (Primescript TM, Dalian, Japan) using 1µg of RNA as template and following the manufacturer’s instructions. Briefly, samples were heated for 10 min at 65 °C. 4µL PrimeScript Buffer 5x, 1µl oligodT (50

µM), 4µl random hexamers and 1µl of PrimeScript RT Enzyme Mix, were sequentially added, followed by a 15 min incubation at 37 °C and 15 s at 85 °C. RNA was removed by incubation with 1µL of RNase H for 20 minutes at 37 °C. Reverse transcription reactions were brought to 200 µl final volume by adding RNase free water, and stored at -20°C. Real-time quantitative PCR amplification (qPCR) was performed using a Rotor-Gene Real-Time PCR System (Labgene Scientific Instruments, Archamps, France). Sequences of primers used in this study are reported in supplementary **Table 2**.

Reactions were incubated at 95°C for 10 min, followed by 40 cycles of denaturation (95°C, 10 s), annealing (58-62 °C depending on the primer sets, 30 s) and elongation (72 °C, 30 s). mRNA expression levels are represented as arbitrary units (A.U.) derived from a standard calibration curve derived from a reference sample. A PCR for each sample was carried out in duplicate for all cDNAs and for the ribosomal protein L19 control. As a further control, qPCR amplicons were analyzed by agarose gel.

Enzymatic analysis

The ventral and dorsal medulla were homogenized in 50 mM TRIS and 1 mM EDTA (pH 7.4), with the addition of 1 mM sodium orthovanadate and 200 µg/ml phenylmethanesulfonyl fluoride. Homogenates were centrifuged at 4000 rpm for 10 minutes at 4°C. Protein concentration was determined in the supernatant using the Bradford protocol and supernatant was used for the following biochemical analyses.

Lipid peroxidation, superoxide dismutase (SOD) activity and CAT activity were performed as described by Nascimento *et al*. (Nascimento *et al.*, 2014) and Ferreira *et al*. (Ferreira *et al.*, 2015)

Briefly, lipid peroxidation was evaluated using MDA levels, using protein mixed with thiobarbituric acid and the measurement was performed at 535 nm and results expressed at nmol/mg protein. SOD determination was performed by reaction of protein and epinephrine. SOD activity was determined by measuring the kinetics of the inhibition of adrenaline auto-oxidation at 480 nm for 1.5 minutes expressed as U/mg protein. For activity of CAT 0.3 M H_2O_2 was added to a mixture containing 200 µg protein and 50 mM phosphate buffer (pH 7.0). The decrease in H_2O_2 was followed by measuring 240 nm absorbance for 3 minutes, and the CAT activity was expressed as U/mg protein.

Statistical analysis.

Bartlett's test was performed to evaluate data homogeneity of the respiratory and sympathetic variables, and statistical results supported the use of a parametric test. Thus, the

significance of the difference between groups was assessed by unpaired Student's t test. The significance level was fixed to $P < 0.05$. The data are expressed as means with associated SEs. Statistical analysis was performed by using GraphPad Prism 5.0 software.

Results

Fig. 1A shows a representative pulsatile arterial pressure (PAP) and HR baseline recordings from 90-days-old in Np and Lp rats. As expected, rats from dams subjected to protein restriction during pregnancy and lactation exhibit increase in mean arterial pressure (Np: 89±7 vs. Lp: 114±6 mmHg, *P*=0.02), but no alteration in HR (*P*>0.05), as describe in Figure 1.

mRNA expression of the genes involved in oxidative stress and glutamatergic genes were analyzed in the medulla of protein-restricted rats. The SOD 2 expression level was down-regulated in medulla of Lp rats (Np: 0.91±0.06 vs. Lp: 0.71±0.04, *P*=0.03, Fig. 2B), but mRNA SOD1 and catalase expression were similar between groups (*P*>0.05, Fig. 2A e C, respectively). In parallel, mRNA expression of Grin1 (*P*>0.05, Fig. 2D), Gria1 (*P*>0.05, Fig. 2E) and Grm1 (*P*>0.05, Fig. 2F) were also similar between Np and Lp group.

In addition, our results showed that MDA levels (nmol/mg protein) were augmented in ventral (Np: 0.25±0.05 vs. Lp: 1.5±0.5 nmol/mg prot, *P*=0.02, Fig. 3A) and dorsal (Np: 0.3±0.05 vs. Lp: 2±0.5 nmol/mg prot, *P*=0.005, Fig. 3B) medulla of Lp group. Moreover, we observed that SOD activity was reduced in ventral medulla of Lp group (Np: 4±0.5 vs. Lp: 2.2±0.6 ativity at U/mg prot, P=0.03, Fig. 3C) in comparison to Np group, but in the dorsal medulla, the SOD activity was similar between groups (P>0.05, Fig. 3D).

Similarly, when the catalase activity was evaluated, we found significant decrease in enzymatic activity in ventral medulla of Lp group (Np: 0.5±0.05 vs. Lp: 0.25±0.03 ativity at U/mg prot, *P*=0.001, Fig. 3E), but the catalase activity in dorsal medulla was similar between Np and Lp group (*P*>0.05, Fig. 3F).

Discussion

As previously reported, rats subjected to protein restriction during pregnancy and lactation exhibited higher levels of baseline arterial pressure at 90-day of age (De Brito Alves, Nogueira, *et al.*, 2014b; Barros *et al.*, 2015). In the present study, we demonstrated that oxidative stress into ventral medulla can be an important underlying mechanism that contribute to the development of arterial hypertension in protein-restricted rats during

gestation and lactation. Our data bring new insights into the etiologic mechanisms underlying the development of arterial hypertension in protein-restricted rats.

We recently reported that rat offspring subjected to protein undernutrition during pregnancy and lactation exhibited sympathetic overactivity before the onset of hypertension (De Brito Alves *et al.*, 2015). In addition, we showed also that at 90 day of age, protein-restricted rats during gestation and lactation exhibit hypertension associated to an increase in the cardiovascular sympathetic tone (Barros *et al.*, 2015). Suggesting that, sympathetic overactivation would contribute for the development of hypertension in this model. However, the mechanisms underlying to the high sympathetic activity and hypertension they were poorly understood.

The sympathetic nervous system is regulated by the brain, especially by the neurons located in rostral ventrolateral medulla (RVLM) in the brainstem (Dampney, 1994; Guyenet, 2006), which determines the basal central sympathetic outflow and also by the neurons located in nucleus of the solitary tract (NTS) that receive inputs from baroreceptors and chemoreceptors periphery, regulating the presympathetic neurons activity of the RVLM (Guyenet, 2006; Zoccal *et al.*, 2008; Moraes *et al.*, 2013).

Recent evidence indicates that oxidative stress into of medulla, especially in the RVLM plays a critical role in the regulation of the sympathetic nervous system and arterial blood pressure in spontaneously hypertensive rats (SHR) (Nishihara, Hirooka, Matsukawa, *et al.*, 2012; Kishi, 2013), as well as in other models, such as salt-induced hypertension (Koga *et al.*, 2008), experimental jet lag (Kishi e Sunagawa, 2011) and renovascular models (two-kidney one-clip) (Oliveira-Sales *et al.*, 2009).

Ferreira e cols., using the same model, observed that the perinatal protein restriction during pregnancy and lactation decreases the activity of several antioxidant enzymes in adult brainstem (midbrain, bridge and medulla) of offspring (Ferreira *et al.*, 2015).

Interestingly, we verified that gene expression of SOD2 (mitochondrial) is down-regulated in the medulla of rats subjected to perinatal protein restriction, but gene expression of SOD1 (citoplasmatic) and catalase it is not changed between Np and Lp group. In addition, we verified also that MDA levels is augmented in ventral and dorsal medulla, but in the ventral medulla we observed that SOD and Catalase activity was decreased in protein-restricted rats. Suggesting that oxidative stress it is more pronounced in ventral medulla and can contribute, in part, to the development of sympathetic overactivation and hypertension observed in rats subjected to protein restriction during pregnancy and lactation.

Previous evidence has demonstrated several mechanisms by which oxidative stress in the ventral medulla, causes sympathoexcitation (Nishi *et al.*, 2013; Chan e Chan, 2014; Sousa *et al.*, 2015). The studies suggest that oxidative stress module the balance between GABA/glutamate in the RVLM (Nishihara, Hirooka, Matsukawa, *et al.*, 2012; Kishi, 2013). For example, higher reactive oxygen species (ROS) in the RVLM enhances glutamatergic neurotransmission, probably by the MAPK signaling pathways (Chan *et al.*, 2003; Chan *et al.*, 2005), and attenuates GABAergic inhibitory inputs to the RVLM, leading to higher sympathoexcitatory inputs to RVLM neurons (Nishihara, Hirooka, Matsukawa, *et al.*, 2012; Chan e Chan, 2014).

Thus, we hypothesize that glutamate in the medulla would be involved in oxidative-stress-evoked sympathoexcitation in rats subjected to protein restriction during pregnancy and lactation. However, our data showed that genes expressions of metabotropic and ionotropic glutamatergic receptors are similar in the medulla in these rats. Nevertheless, it is important to highlight hypothesis still requires further experimental verification, through of analysis of glutamatergic gene expression on RVLM as well as by the microinjection experiments.

On the other hand, it has been suggest that ANG II plays also an important role in autonomic efferent activity by acting on RVLM neurons via AT1 receptor (Chan *et al.*, 2007; Nishi *et al.*, 2013). Previous reports have shown that an augmentation of ROS dependent of NADPH oxidase activation leads to a long-term pressor response to Ang II via transcriptional up-regulation of AT1 mRNA expression (Chan *et al.*, 2007; Braga, 2013; Nishi *et al.*, 2013). This signaling pathway is worthy of future investigation in protein-restricted rats during pregnancy and lactation.

In addition, previous studies have demonstrated that epigenetic process would induce modification in gene expression involved in the oxidative status (Bhusari *et al.*, 2010; Yara *et al.*, 2015). For example, higher levels of DNA methylation or higher methiltransferase activity it is associated with decrease expression in SOD expression (Bhusari *et al.*, 2010; Yara *et al.*, 2015). We suggest that probably, higher DNA methylation can leads to the SOD down-regulation in the medulla of rats subjected to protein restriction during pregnancy and lactation.

In conclusion, the present study suggests that offspring from protein-restricted dams exhibit enhanced arterial blood pressure combined with imbalance oxidative in medulla ventral. These findings represent new insights into the mechanisms underlying the arterial blood pressure control of rats that have undergone perinatal protein restriction and can aid in

understanding why blood pressure increases in individuals subjected to protein undernutrition during a critical period of life.

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Figure and Table Captions

Figure 1. **Protein-restricted rats during pregnancy and lactation exhibit higher arterial blood pressure at 90 days age**. Panel A shows representative tracing of pulsate arterial pressure (PAP) and heart rate (HR). Evaluation of MAP (panel B) and HR (panel C) at rest. Gray bars represent Np group (17% protein, n=8) and white bars Lp group (8% protein, n=8). All pups were fed a standard chow diet at weaning. Values are mean±SEM (*P<0.05, Student's t-test)

Figure 2. **Protein-restricted rats during pregnancy and lactation exhibit loss mRNA expression of SOD2 in medulla at 90 days age.** Evaluation of mRNA of SOD1 (panel A), SOD2 (panel B), catalase (panel C), Grin 1 (panel D), Gria 1 (panel E) and Grm 1 (panel F) in medulla**.** Gray bars represent Np group (17% protein, n=5) and white bars Lp group (8% protein, n=5). All pups were fed a standard chow diet at weaning. Values are mean±SEM (*P<0.05, Student's t-test)

Figure 3. **Protein-restricted rats during pregnancy and lactation exhibit higher MDA levels in ventral and dorsal medulla and decrease in SOD and catalase activity in ventral medulla at 90 days age.** Evaluation of MDA levels (panel A and B), SOD activity (panel C and D), and catalase activity (panel E and F) in ventral and dorsal medulla**.** Gray bars represent Np group (17% protein, n=8) and white bars Lp group (8% protein, n=8). All pups were fed a standard chow diet at weaning. Values are mean±SEM (*P<0.05, Student's t-test)

Table 1. Nutritional Composition of the experimental diets (g/100g diet)

Table 2. Primers sequence used to perform qRT-PCR.

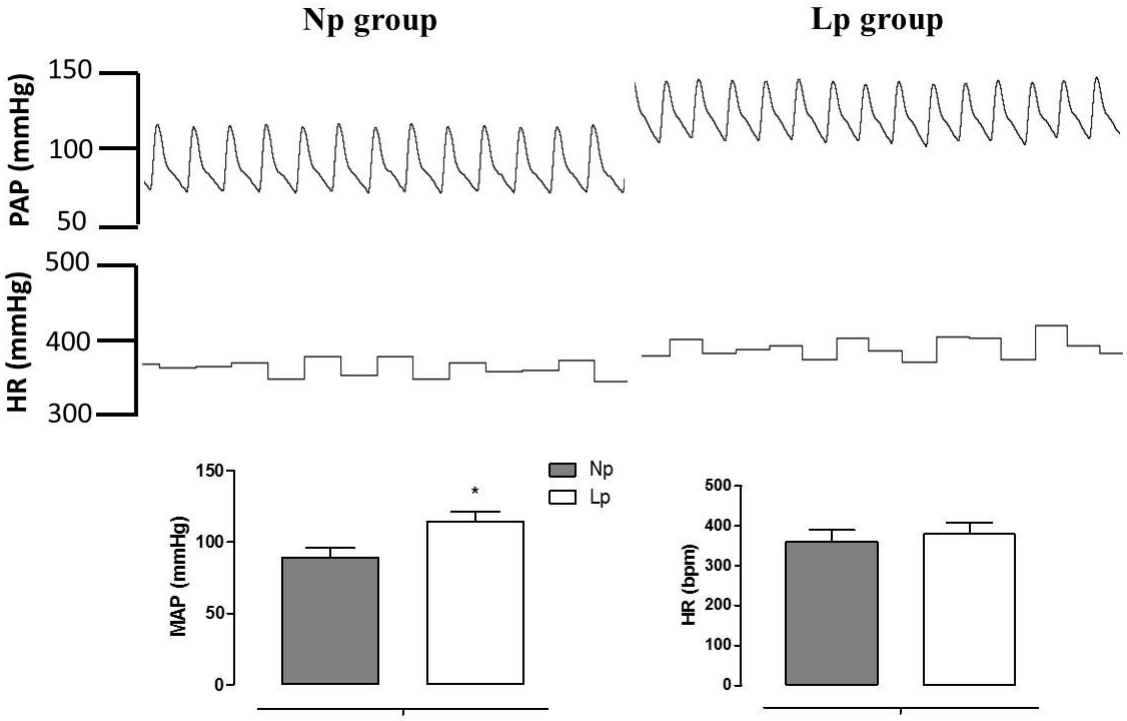
Np group
Lp group
PAP (mmHg)
150
100
50
HR (mmHg)
500
400
300
MAP (mmHg)
150
100
50
0
*
Np
Lp
HR (bpm)
500
400
300
200
100
0

Figure 1.

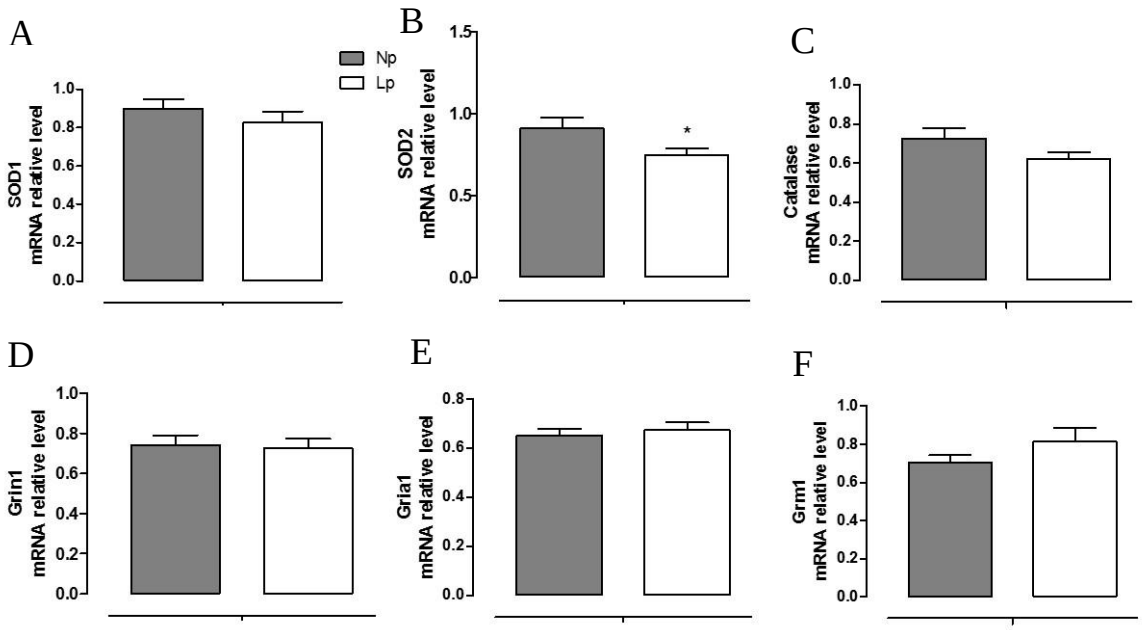
A
B
C
D
E
F
Np
Lp
SOD1 mRNA relative level
SOD2 mRNA relative level
Catalase mRNA relative level
Grin1 mRNA relative level
Gria1 mRNA relative level
Grm1 mRNA relative level
*

Figure 2.

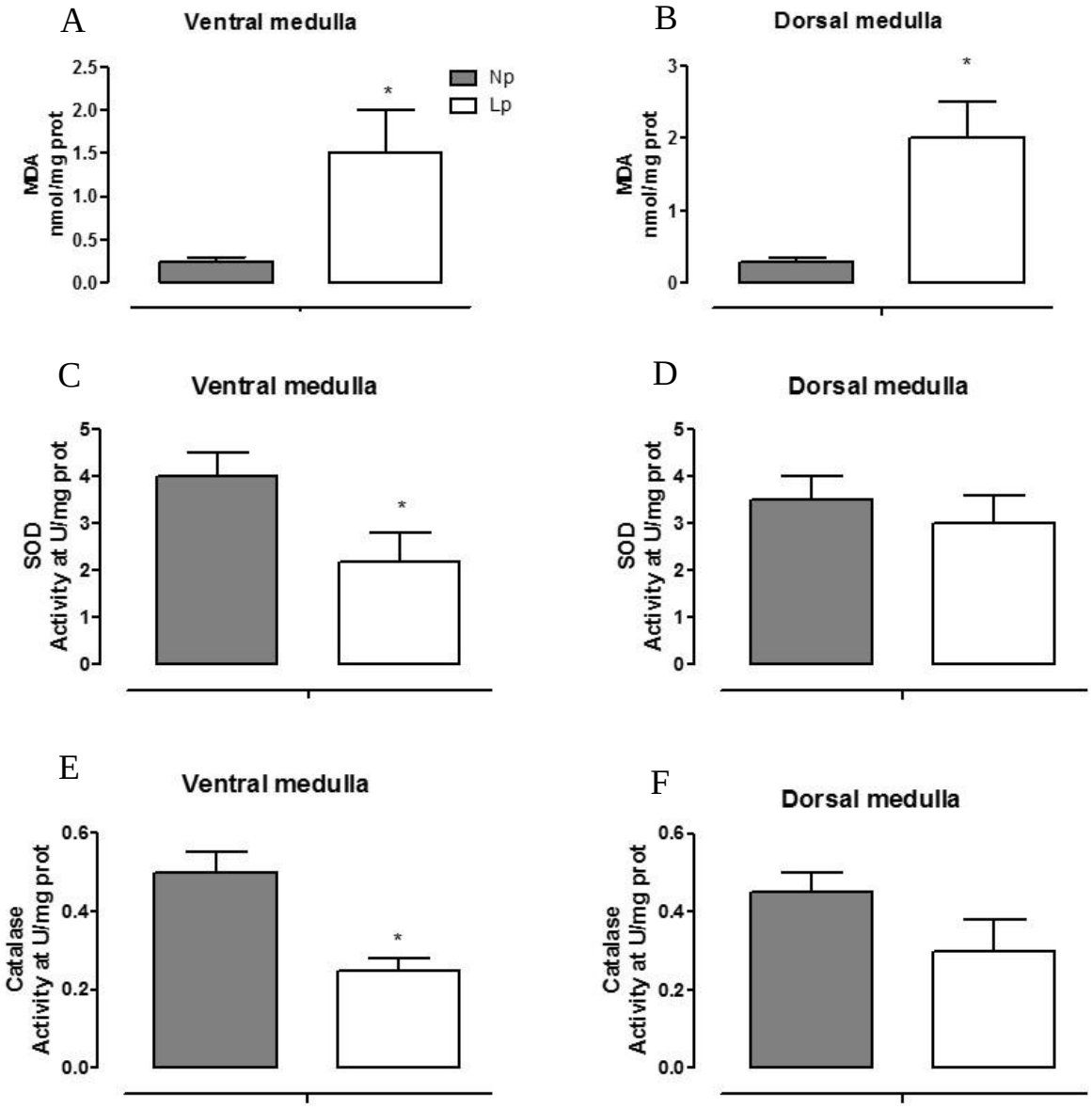
A
Ventral medulla
MDA
nmol/mg prot
Np
Lp
B
Dorsal medulla
MDA
nmol/mg prot
C
Ventral medulla
SOD
Activity at U/mg prot
D
Dorsal medulla
SOD
Activity at U/mg prot
E
Ventral medulla
Catalase
Activity at U/mg prot
F
Dorsal medulla
Catalase
Activity at U/mg prot

Figure 3.

Supplemental Table 1. Nutritional Composition of the experimental diets (g/100g diet)

Nutrient	Normal protein (17% protein)	Low protein (8% protein)
Casein (85%)*	20	9.41
Dextrin cornstarch	13	13.2
Cellulose	5	5
Sucrose	10	10
Cornstarch	39.74	50.34
Soybean oil	7	7
Choline	0.25	0,25
Methionine	0.3	0.3
Vitamin mix	1	1
Mineral mix	3.5	3.5
Energy density (kJ/g)	16.26	16.26

* The casein used in preparation of diet was 85% purity.

Table 2. Primers sequence used to perform qRT-PCR

Gene	Foward/Reverse	Tm	Sequence 5'-3'	Amplicon size, bp
Catalase	F	60°C	CATCGGCACATGAATGGCTA	281pb
	R		ACCTTGGTCAGGTCAAATGG	
SOD 1	F	58°C	TGAAGAGAGGCATGTTGGAG	164pb
	R		CCACCTTTGCCCAAGTCATC	
SOD 2	F	60°C	TCATGCAGCTGCACCACAGC	138pb
	R		CCATTGAACTTCAGTGCAGG	
Grin 1	F	60°C	TACAACTGGAACCACATCATCC	145 pb
	R		TGTCATAGGACAGTTGGTCGAG	
Gria 1	F	60°C	TGTTTGCCTACATTGGAGTGAG	149 pb
	R		GAACCACAGGCTGTTGAATATG	
Grm 1	F	60°C	CTATCATAGCCATCGCCTTTTC	143 pb
	R		GAGGAAAATACCAGCCAGAATG	
RPL19	F	58°C	CTGAAGGTCAAAGGGAATGTG	195pb
	R		GGACAGAGTCTTGATGATCTC	

ARTIGO 05 – ARTIGO A SER SUBMETIDO AO AMERICAN JOURNAL OF PHYSIOLOGY: REGULATORY, INTEGRATIVE AND COMPARATIVE PHYSIOLOGY

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ARTIGO 05: Carotid body denervation improves arterial blood pressure in protein-restricted rats offspring

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Running title: Perinatal low-protein diet and carotid body denervation

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Abstract

Purpose: We eliminated carotid body inputs in rats exposed to low protein diet (LP-cbd group) to test the hypothesis that heightened peripheral chemoreceptor activity contributes to development of hypertension in protein-restricted rats.

Methods: Male Wistar rats were subjected to control (NP, 17% of casein) or low-protein (LP, 8% of casein) diet during gestation and lactation. At 28 days of age, the carotid sinus nerves were surgically denervated under general anaesthesia. Sham-operated rats (NP-sham and PR-sham) underwent the same surgical procedures to expose the CB but the carotid sinus nerves were left intact. Direct measurements of arterial pressure (AP), heart rate (HR) and respiratory frequency (Rf) were recorded from the awake male offspring at the 90th d old. It was used KCN (0.04 %, 100uL/rat) for evaluate the carotid body sensitivity and hexamethonium (25 mg/kg) for sympathetic sensitivity.

Results: CB denervation in LP rats significantly reduced the arterial pressure (approximately 20 mmHg). When compared to the LP rats, the improvement in arterial pressure of LP-cbd rats was associated with decrease of sympathetic tone, elicited by decrease of low frequency (LF) variability (approximately 58%, $P<0.05$), pressor response attenuation after administration of the ganglionic blocker (approximately 29%, $P<0.05$) and probably improvement in baroreflex sensitivity (approximately 76%). Moreover, unanesthetized LP rats showed enhanced pressor ($P<0.05$) response to peripheral chemoreflex activation in comparison to NP rats.

Conclusion: Carotid body are, in part, responsible for elevated sympathetic and arterial blood pressure in rats subjected to protein restriction during pregnancy and lactation.

Key words: protein-restriction, arterial hypertension, peripheral chemoreflex, carotid body denervation.

Introduction

Adverse events experienced *in utero* or during the perinatal life (gestation, lactation and first infancy) has been propose as predisposing factor to the development of arterial hypertension and metabolic diseases in later life (Langley-Evans *et al.*, 1996; Costa-Silva *et al.*, 2009; De Brito Alves, Nogueira, *et al.*, 2014b; Tennant *et al.*, 2014; Barros *et al.*, 2015). Although a relationship between maternal protein restriction during pregnancy and lactation and the development of arterial hypertension in offspring at adult life has been previously described, its underlying mechanisms are poorly understood.

Recently, our laboratory reported that rats subjected to maternal protein restriction during pregnancy and lactation exhibit enhanced arterial blood pressure and sympathetic overactivity combined with amplified ventilatory-autonomic responses to peripheral chemoreflex activation (De Brito Alves, Nogueira, *et al.*, 2014b; Barros *et al.*, 2015). These latter finding demonstrate that peripheral chemoreceptors inputs can be involved in the establishment and maintenance of hypertension in adult life of protein-restricted rats.

The main peripheral chemoreceptor site in rats is the carotid body (CB) located at the bifurcation of the common carotid artery (Gonzalez *et al.*, 1994; Feldman *et al.*, 2003). The CB chemoreceptors play a main function in respiratory and cardiovascular homeostatic control, since their activation by hypoxic stimuli induces sympathetic-respiratory overactivity and enhancement of blood pressure (Costa-Silva *et al.*, 2012; Prabhakar, 2013).

Clinical and experimental evidence indicates that CB chemoreceptors exhibit an important function in pathophysiology of hypertension (Trzebski *et al.*, 1982; Franchini e Krieger, 1993; Abdala *et al.*, 2012). For example, spontaneously hypertensive rats (SHR) and rats exposed to intermittent hypoxia present hypertension associated with amplified response to peripheral chemoreceptors activation (Zoccal *et al.*, 2008; Ding *et al.*, 2011; Zoccal e Machado, 2011; Abdala *et al.*, 2012).

Surprisingly, recent reports have shown that the ablation of CB might be used as therapeutic measure to reduce arterial blood pressure. For example, carotid body denervation (CBD) in SHR reduces sympathetic activity and arterial pressure (Mcbryde *et al.*, 2013; Marcus *et al.*, 2014). In addition, clinical studies have shown that the ablation of CB by hyperoxia reduce blood pressure in patients with resistant hypertension, as well as improve the cardio-respiratory control of patients with congestive heart failure (Niewinski *et al.*, 2013; Marcus *et al.*, 2014). This findings suggest that CBD could be an effective antihypertensive treatment.

Despite the detected association between chemoreflex sensitivity, sympathetic overactivity and hypertension, the hypothesis that peripheral chemoreceptor drive plays a crucial role in the maintenance of high arterial pressure in protein-restricted rats remains undetermined. Based on these evidences, we hypothesized that selective CB denervation applied before onset the hypertension in protein-restricted rats would improve autonomic sympathetic tone and induce an attenuation in the arterial pressure in adulthood.

Materials and methods

The experimental protocol was approved by the Ethical Committee of the Biological Sciences Centre (protocol 23076 019345/2013-81), Federal University of Pernambuco, Brazil. All efforts were made to minimize animal discomfort and the number of animals used; in addition, we followed the Guidelines for the Care and Use of Laboratory Animals.

Animals and experimental groups

Virgin female albino Wistar rats (*Rattus novergicus*) were obtained from the Academic Centre of Vitoria de Santo Antão (CAV), Federal University of Pernambuco, Brazil. Animals were maintained at room temperature of 22 ± 1^{o}C with controlled light–dark cycle (dark 18:00–06:00 hours). Standard laboratory chow (52% carbohydrate, 21% protein, and 4% lipids - Labina®, Purina Agriband, São Paulo, Brazil) and water were given *ad libitum* up to the 3-month, when rats were mated (2 females for 1 male). The day on which spermatozoa were identified in vaginal smear was considered as the conception and the pregnant rats were transferred to individual cages: mothers fed with 17% casein diet (n=5, normal protein group, NP) and mothers fed with 8% casein diet (protein-restricted group, PR, n=5) and water *ad libitum*. Both diets were isoenergetic (**Table 1**) and were offered during pregnancy and lactation. The low protein diet differed from the normal protein diet in the content of protein and carbohydrate. Diets were elaborated at the Laboratory of Experimental Nutrition-CAV, Federal University of Pernambuco, according to the American Institute of Nutrition – AIN-93 (Reeves *et al.*, 1993). The casein was previously analysed and showed 85% of purity (85g of protein for each 100g of casein).

During the suckling period, the offspring were maintained as litters of eight pups randomly. At weaning, three or four male offspring of each litter were randomly housed in collective cages and received a standard diet and water *ad libitum*. Four experimental groups were designed according to the diet manipulation and carotid body denervation (CBD): NP-

sham (sham-operated, n=10), NP-cbd (carotid body denervation, n=10), PR-sham (n=8) and PR-cbd (n=12).

The experimental groups were formed with two or three rats from each mother. Experiments of carotid body denervation was performed at 28-day and cardiovascular and respiratory evaluations in vivo were performed in 90-days.

Carotid body denervation

At 28 days old, rats were anaesthetised with ketamine (80 mg/kg) and xylazine (10 mg/kg). Using sterile techniques, an anterior midline neck incision was performed and the sternohyoid and sternocleidomastoid muscles were carefully retracted. The carotid bifurcation was exposed, the occipital artery was retracted, the CB visualised and the carotid sinus nerve and its branches sectioned. Sham-operated rats underwent the same surgical procedures to expose the CB but the carotid sinus nerves were left intact. To assess the completeness of CSD, arterial pressure and heart rate responses was recorded after I.V. injection of sodium cyanide (KCN, 100 μl of 0.04%) 8 weeks post CSD.

Cardiovascular and respiratory evaluations *in vivo*

One day before of the experiments, NP-sham (n=10), PR-sham (n=8) and PR-cbd (n=12) rats were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg), and the femoral artery and vein were cannulated (PE-50 connected to PE- 10; Clay Adams, Parsippany, NJ, USA). The catheters were filled with heparinized saline (NaCl 0.9%), tunneled subcutaneously and exteriorized through the back of the neck. After surgery, the animals received injection of ketoprofen (5 mg/kg ip) and a period of 24 hours were allowed until rats fully recovered from the surgical and anaesthetic procedures. After the next day, mean arterial pressure (MAP) and heart rate (HR) were recorded in unanesthetized freely-moving animals by connecting the arterial catheter to a pressure transducer. The signals were amplified (ML866/P, ADInstruments, Power Lab, Bella Vista, NSW, Australia), sampled at 2 kHz, digitalized (Power Lab, model 4/30, ADInstruments) and digitalized using appropriate software (LabChart7 Pro, ADInstruments). Recordings of baseline pulsatile arterial pressure (PAP), mean arterial pressure (MAP) and heart rate (HR) were performed for 50 min. After 50 min of acclimatization and cardiovascular recordings, measurements of respiratory frequency (RF) were also performed using the whole body plethysmography method (Malan, 1973). Before recording baseline data, animals were placed into the plexiglas chamber (5 L), which was flushed with humidified room air and temperature at 25°C. After this

acclimatization period, Rf was recorded when airflow was suspended for short periods (3 min); the pressure oscillations caused by breathing were captured by a pressure differential transducer connected to a signal amplifier (ML141 Spirometer, PowerLab, ADInstruments). Then, the signals were captured into an acquisition system and data analysis (PowerLab, ADInstruments). All data were analysed off-line using appropriate software (LabChart 7 Pro, ADInstruments).

After baseline recordings of respiratory frequency and arterial pressure, the peripheral chemoreflex was activated by intravenous injection of potassium cyanide (KCN; 40 μg/100 μl per rat; Merck, Darmstadt, Germany) accordingly with previous reports (Franchini e Krieger, 1993; Machado e Bonagamba, 2005). At the end of experiments, rats were euthanized with overdose of ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). For overdose was used approximately 1 mL of ketamine-xylazine mixture.

Spectral Analyses

An indirect evaluation of autonomic modulation of vascular resistance and cardiac function was performed through the analysis of variability of arterial pressure and heart rate in the frequency domain (Zoccal *et al.*, 2009). Oscillations of arterial pressure and heart rate at low-frequency range (LF) are representative of the modulatory effects of sympathetic activity controlling vascular tonus and heart activity, while oscillations at high-frequency range (HF) are associated with a respiratory or parasympathetic modulation of blood vessels and the heart, respectively (Malliani *et al.*, 1991a; Bernardi *et al.*, 2001; Zoccal *et al.*, 2009). To reach this goal, beat-by-beat time series of systolic arterial pressure (SAP) and HR were extracted from baseline cardiovascular recordings (10 min epochs) of pulsatile arterial pressure of NP and LP rats (Chart Pro, ADInstruments, NSW, Australia) and the overall variability of these series was assessed using Fast Fourier Transform (FFT) spectral analysis (Cardioseries Software v2.4, available on https://www.sites.google.com/site/cardioseries/home) (Tezini *et al.*, 2013). The power of the oscillatory components obtained from rats of NP and LP groups was quantified in two frequency bands: LF (0.20–0.75 Hz) and HF (0.75–3.0 Hz) (Cerutti *et al.*, 1991; Zoccal *et al.*, 2009).

Evaluation of sympathetic tonus on the vascular system

The sympathetic vascular tone was evaluated by an intravenous injection of hexamethonium (25 mg/kg, Sigma, St Louis, MO, USA) and calculated by difference between the MAP after the blocker and the baseline MAP.

Spontaneous baroreflex sensitivity

In this study, the spontaneous baroreflex sensitivity was calculated through sequence method by computer software CardioSeries (version 2.4).

Statistical Analysis

Each experimental group included two animals from each litter. Data sets (10min) were used for analysis of mean pulse pressures and heart rate. Values are presented as mean±SEM. Data were compared by two-way ANOVA followed by Newman–Keuls multiple comparison test. For analysis between NP and LP group was used the t-test student. Differences between groups with P values of <0.05 were considered significant. Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA).

Results

Effect of CBD on arterial blood pressure

The representative pulsatile arterial pressure (PAP), MAP and HR baseline recordings from 90-days-old in NP-sham, LP-sham and LP-cbd rats are shown **in Fig. 1a**. As demonstrated previously by our laboratory, LP-sham rats exhibited increase in arterial pressure compared to NP-sham group (P<0.05; **Fig. 1b**). CBD conducted before the onset of increase in the arterial pressure was capable of reduce the arterial blood pressure in LP rats (P<0.05; **Fig. 1b**). However, the HR baselines were similar between in all groups (P>0.05; **Fig. 1c**).

Effect of CBD on sympathetic tone

As observed in the **Fig. 2a**, LP-csd rats exhibited an attenuation in magnitude of oscillatory components at LF range of systolic arterial pressure (P<0.05) when compared to the LP-sham rats. However, HF components of the SAP (P>0.05; **Fig. 2 b**) and LF/HF ratio of the PI were similar between groups (P>0.05; **Fig. 2 c**).

Fig. 3 a show a representative PAP and MAP recordings during ganglionic blocker with hexamethonium. As previously shown, after the administration of a ganglionic blocker, the delta AP variation was larger in the LP group (P<0.05; **Fig 3b**). CBD induced an attenuation in the delta AP variation, suggesting an improvement in sympathetic tone in LP-cbd rats (P<0.05; **Fig. 3 b**)

Effect of CBD on spontaneous baroreflex sensitivity

As previously shown, LP and NP rats exhibited similar gain of the baroreflex, but LP-cbd rats exhibited a tendency in the improvement of baroreflex measured by sequence analysis (P=0.08; **Fig. 2 d).**

Effect of CBD on peripheral chemosensitivity

The **fig. 4 a** show PAP, MAP recordings during peripheral chemoreflex activation. The peripheral chemoreflex actvation response was absent in LP-csd, but produced pressor, bradycardic and tachypnoeic responses in both NP and LP groups (**Fig. 4a**). LP rats shown high response pressoric (P<0.05; **Fig. 4b**) in comparison to NP group. However, the tachypneic response (P=0.14; **Fig. 4c**) the magnitude of decrease in the HR were similar between the groups (P=0.60; **Fig. 4d**).

Discussion

As previously reported, rats subjected to protein undernutrition during pregnancy and lactation exhibited higher levels of baseline arterial pressure and sympathetic overactivity at 90-day of age (De Brito Alves, Nogueira, *et al.*, 2014b; Barros *et al.*, 2015). Our results confirm a role for the CB chemoreflex in the aetiology of arterial hypertension in protein-restricted rats. In the present study, we demonstrated that ablation of CB chemoreceptors is effective in reducing of sympathetic tone and in improving arterial blood pressure of rats exposed to maternal protein-restriction. Cumulatively, these results support the notion that the CB chemoreceptors contributes importantly to augmentation in the arterial blood pressure in our maternal protein-restriction model.

Interestingly, different reports have shown that the reduction in the arterial pressure of CBD models is associated with improvement of sympathetic tone. For example, CB chemoreceptors ablation in SHR and rats with congestive heart failure induce improvement of arterial blood pressure and autonomic sympathetic tone (Abdala *et al.*, 2012; Mcbryde *et al.*, 2013; Marcus *et al.*, 2014). Thus, ablation of CB chemoreceptors has been propose as putative therapeutic target for the treatment of arterial hypertension (Del Rio *et al.*, 2013; Mcbryde *et al.*, 2013; Ribeiro *et al.*, 2013; Marcus *et al.*, 2014).

Previous stuides from our laboratory have shown that LP rats exhibit imbalance in the cardiac autonomic control associated to increased activity of the sympathetic nervous system (De Brito Alves, Nogueira, *et al.*, 2014b; Barros *et al.*, 2015). In this work, we verified that

the variability at low-frequency range of SAP, which exhibit correlation with sympathetic drive to blood vessels and to the heart, was reduced in LP-cbd rats. Moreover, ganglionic blockade with hexamethonium was assessed for evaluate the relative level of sympathetic tone in LP-cbd rats. Clearly, we observe that the delta AP, after hexamethonium infusion was attenuated in LP-cbd group, suggesting that sympathetic vascular activity in these animals was attenuated, which can contribute for the improvement of their arterial pressure.

It is very important to highlight that arterial pressure control involve multiples mechanisms. For example, it was previously shown that rats subjected to maternal protein restriction exhibit higher blood pressure associated with deficit in the glomerulogenesis and lower nephron numbers than their normoproteic (Villar-Martini *et al.*, 2009; Vieira-Filho *et al.*, 2014). Interesting, in the present study, we showed that CB chemoreceptors play an important function in the augmentation of arterial pressure in protein-restricted rats, since that LP-cbd rats exhibited pressoric levels similar to the NP group.

Moreover, we found that LP-cbd rats exhibit a tendency in improvement spontaneous baroreflex sensitivity. However, additional studies will be necessary to confirm the effect of CB denervation in rats subjected to perinatal protein restriction, because in the present study we assess only baroreflex sensitivity. It is well established that the baroreflex control is crucial in short term control of arterial blood pressure; and reduced baroreflex sensitivity is associated with increase in arterial pressure and development of hypertension (Kanbar *et al.*, 2008; Carthy, 2014). However, on some hypertension models the baroreflex function no are changed (Zoccal *et al.*, 2009; Barros *et al.*, 2015).

Previous studies have showed that CB denervation provided an improvement in baroreflex function (Abdala *et al.*, 2012; Mcbryde *et al.*, 2013). Apparently, SHR and rats with congestive heart failure exhibit improvement of arterial blood pressure associated with improvement of baroreflex function (Abdala *et al.*, 2012; Mcbryde *et al.*, 2013; Marcus *et al.*, 2014). However, the underlying mechanisms by which CB denervation improves baroreflex sensitivity are poorly understood.

The maturation of peripheral chemoreceptors occurs after birth (De Caro *et al.*, 2013; Kim, 2013). Thus, insults during fetal or postnatal life can disrupt morph-functional status of CB chemoreceptors (Peyronnet *et al.*, 2007). Recently, we shown that prior to the development of hypertension, juvenile rats subject to protein-restriction during pregnancy and lactation exhibit enhanced sensitization of peripheral chemoreflex (De Brito Alves, Nogueira, *et al.*, 2014b).

Thus, we suggest that the increase in arterial blood pressure of offspring from dams submitted to protein restriction during pregnancy and lactation are dependent, at least in part, on the sensitization of peripheral chemoreflex. However, the mechanisms by which protein-restricted rats during pregnancy and lactation exhibit high peripheral chemoreflex sensitization are not fully understood. So, futures studies are still requires for understand mechanistically as maternal low protein diet induce changes in peripheral chemosensitivity in the offspring the short and long-term.

We have used indirect measures for evaluation of autonomic sympathetic tone. However, additional studies will be needed to verify more directly the effects of CBD in protein-restricted rats on sympathetic activity and autonomic control of the vasculature in rats subject to low protein diet during pregnancy and lactation.

Nevertheless, our data bring new insights into the etiological mechanisms underlying the development of arterial hypertension in protein-restricted rats; we suggest a critical role of the peripheral chemoreceptors.

In conclusion, the present study suggests that CB denervation in offspring from protein-restricted dams prior to the establishment of hypertension improves autonomic sympathetic tone, baroreflex and reduce arterial blood pressure. These findings can help in understanding why blood pressure increases in rats subjected to protein undernutrition during a critical period of life

Authors contributions statement

J.L.B.A., V.O.N. and M.A.B contributed to experiments with conscious rats. J.L.B.A, J.H.C.S., A.G.W. and C.G.L. contributed to the conception and experimental design, data analyses and interpretation of the findings and the preparation of this manuscript. All authors read and approved the final manuscript.

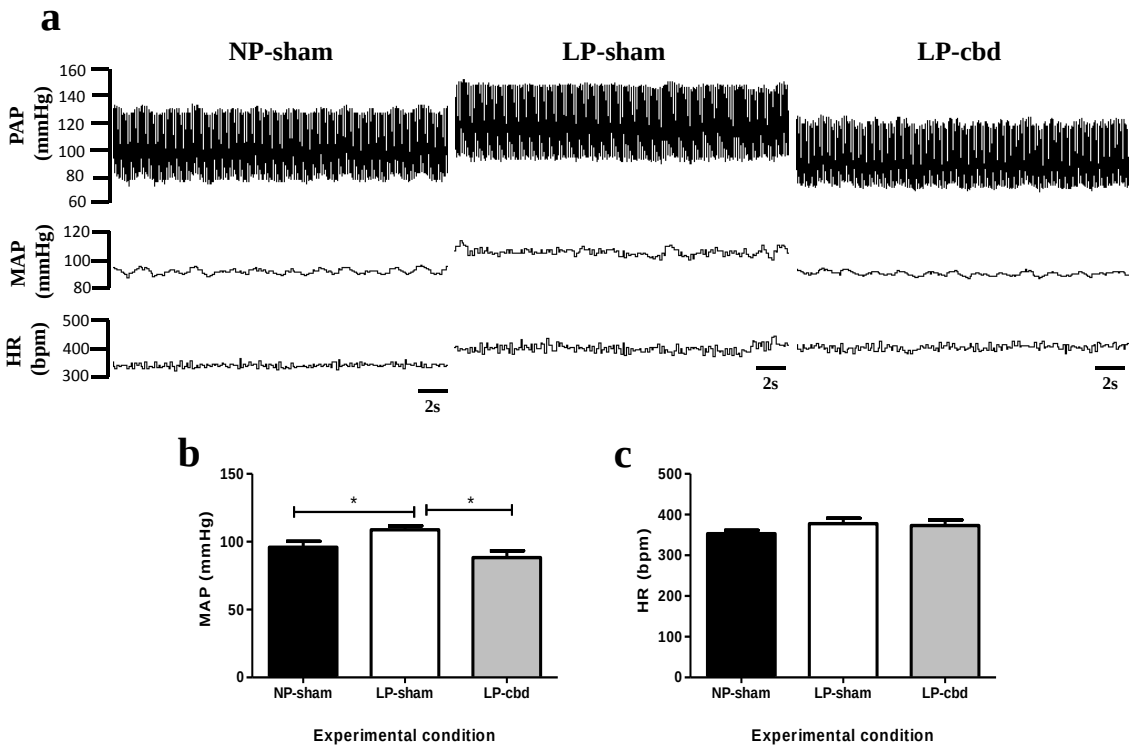

Fig. 1. CBD bilateral improve arterial blood pressure in protein-restricted rats. (a) representative tracing of pulsatile arterial pressure (PAP), mean arterial pressure (MAP) and heart rate (HR) at rest. Evaluation of MAP (b) and HR (b) of 90-d-old male rat pups (sham or cbd) of dams fed an NP or an LP diet during pregnancy and lactation. All pups were fed a standard chow diet at weaning. Values are means ± SEMs, n = 8–12. * $P < 0.05$ (two-way ANOVA).

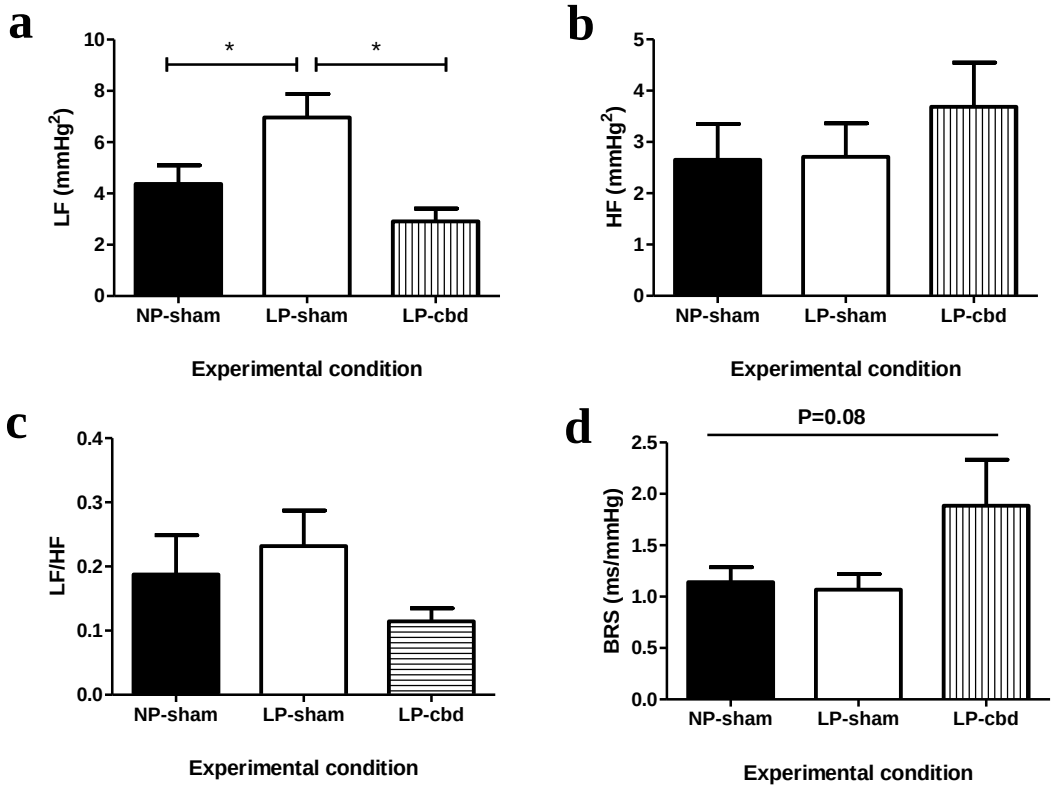

Fig. 2. CBD bilateral improve magnitude of oscillation at the LF range in protein-restricted rats. Evaluation of average magnitudes of LF (a) and HF (b) components of SAP, LF/HF index of PIs (c) and spontaneous baroreflex (d) of 90-d-old male rat pups (sham or cbd) of dams fed an NP or an LP diet during pregnancy and lactation. All pups were fed a standard chow diet at weaning. Values are means ± SEMs, n = 8–12. * $P < 0.05$ (two-way ANOVA).

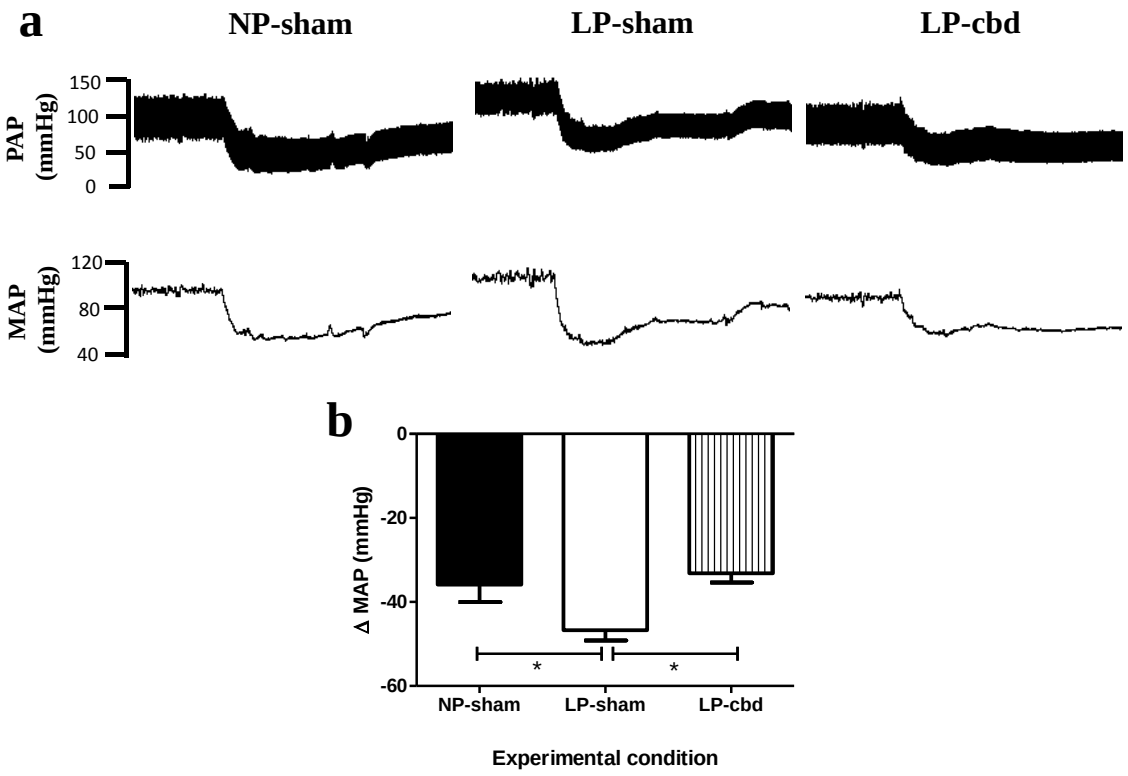

Fig. 3. CBD bilateral improve sympathetic tonus in protein-restricted rats. (a) Representative recordings of the pulsatile arterial pressure (PAP) and mean arterial pressure (MAP) after hexamethonium. Evaluation of delta change of the MAP (b) of 90-d-old male rat pups (sham or cbd) of dams fed an NP or an LP diet during pregnancy and lactation. All pups were fed a standard chow diet at weaning. Values are means ± SEMs, n = 8–12. * $P < 0.05$ (two-way ANOVA).

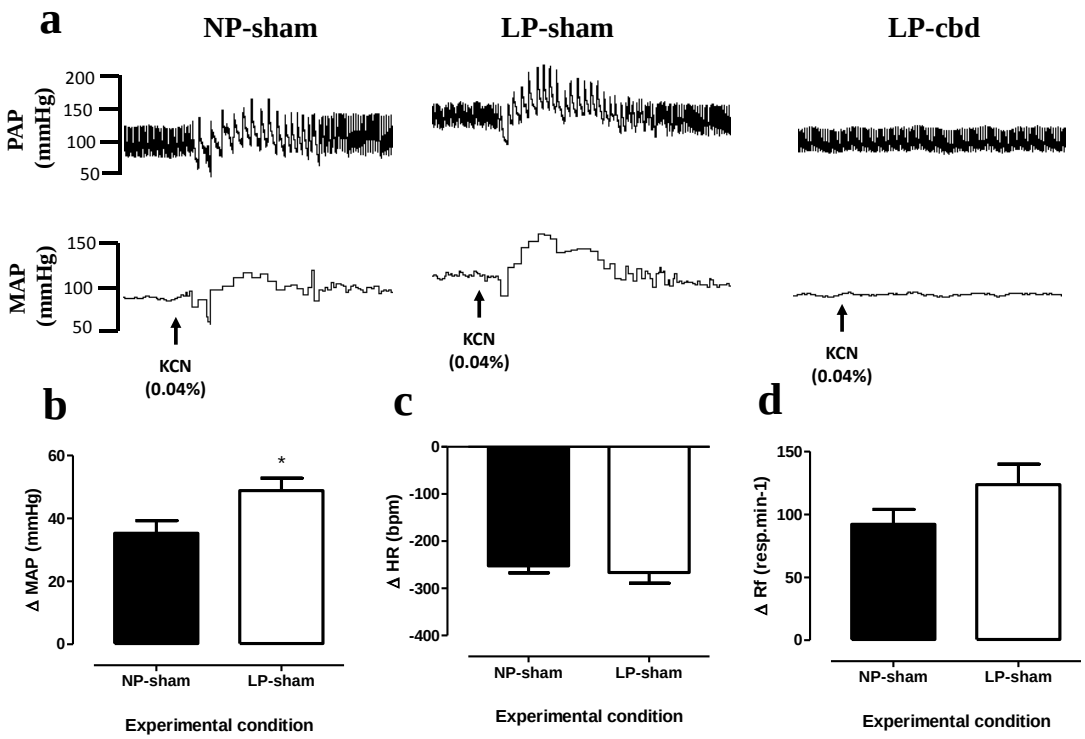

Fig. 4. Maternal protein restriction induce increase in peripheral chemosensitivity. (a) Representative tracing of pulsatile arterial pressure (PAP), mean arterial pressure (MAP) during peripheral chemoreflex activation. Evaluation of delta change of the MAP (b), HR (b) and respiratory frequency (Rf) of 90-d-old male rat pups (sham or cbd) of dams fed an NP or an LP diet during pregnancy and lactation. All pups were fed a standard chow diet at weaning. Values are means ± SEMs, n = 8–12. *Different from NP ($P<0.05$; unpaired Student's t test).

Supplemental Table 1. Nutritional Composition of the experimental diets (g/100g diet)

Nutrient	Normal protein (17% protein)[1]	Low protein (8% protein)
Casein (85%)*	20	9.41
Dextrin cornstarch	13	13.2
Cellulose	5	5
Sucrose	10	10
Cornstarch	39.74	50.34
Soybean oil	7	7
Choline	0.25	0,25
Methionine	0.3	0.3
Vitamin mix	1	1
Mineral mix	3.5	3.5
Energy density (kJ/g)	16.26	16.26

* The casein used in preparation of diet was 85% purity.

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ARTIGO 06 – ARTIGO A SER SUBMETIDO AO THE JOURNAL OF NUTRITION

Informações da revista: Fator de impacto: 4.2; Qualis A1 – MEDICINA II

ARTIGO 06: Perinatal protein restriction induces altered expression of metabolic genes in skeletal muscle and liver of young and adult male offspring

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Conflict of interest statement

The authors declare no competing financial interests.

Abstract

Background: Exposure to a perinatal protein restriction induces persistent physiological alterations and predisposes to insulin resistance, type 2 diabetes and cardiovascular diseases in the offspring.

Objectives: The aims of the study were to evaluate the effects of a perinatal low-protein diet on the expression of genes involved in glucose and fatty acid metabolism in oxidative (soleus) and glycolytic (EDL) skeletal muscles and liver.

Methods: Wistar rat dams were fed a control [normal-protein (Np); 17% protein] or a Lp (8% protein) diet during pregnancy and lactation, and their male offspring were studied at 30 d and 90 d of age. Male offspring (Np: n=5; Lp: n=5) were euthanized and soleus and EDL muscle and liver were collected. Expression levels of HK2, PFK, PDK4, CPT1, βHAD, CS, PGC1a, PEPCK, G6Pase, and FAS genes and proteins were determined by real time-PCR and western blotting, respectively.

Results: At 30 d of age, mRNA and protein expression of PDK4 and mRNA expression of βHAD were downregulated in soleus of Lp pups. In EDL muscle, Lp rats showed decreased in mRNA expression of HK2, PFKM, CS and PGC1a. Protein expression of HK2 and PFKM were also reduced in Lp rats. In the liver, gene expression for G6Pase, βHAD and CS were upregulated in Lp rats. At 90 d of age, mRNA expression of PDK4 was downregulated in soleus and EDL muscle. In liver, mRNA for FAS it was upregulated, but βHAD and PGC1a downregulated in Lp group.

Conclusion: Maternal protein restriction induces age-dependent alterations in metabolic gene expression patterns involved in glucose and lipid metabolism in skeletal muscle and liver of male offspring. These alterations might contribute to the development of metabolic dysfunctions later in life.

Introduction

Epidemiological, clinical and experimental evidences have shown that insults as malnutrition during perinatal period is a significant predisposing factor for the development of insulin resistance, type 2 diabetes (T2D), hypertension, and other cardiovascular diseases in offspring later life (Fernandez-Twinn *et al.*, 2006; De Brito Alves, Nogueira, *et al.*, 2014b).

Maternal low-protein diet during gestation and/or lactation is one of the most extensively studied animal models of phenotypic plasticity, which refers to the ability of a single genotype to produce variable behavioral, morphologic, and/or physiologic phenotypes in individuals in response to different environmental circumstances encountered during development (West-Eberhard, 2005). It was shown that offspring from protein-restricted dams (8% protein) during gestation and lactation exhibit long-lasting growth restriction (Orozco-Solis *et al.*, 2011), age-dependent loss of glucose tolerance (Ozanne *et al.*, 1996; Leandro, Fidalgo, *et al.*, 2012), insulin resistance (Muhlhausler *et al.*, 2009; Dunlop *et al.*, 2015) and hypertension (De Brito Alves, Nogueira, *et al.*, 2014b; M.A.V. Barros, 2014).

It has been shown that the enhanced metabolic disease susceptibility as insulin resistance and T2D would be linked with impaired structure and function of several organs, including brain (Grace *et al.*, 2011), liver (Jia *et al.*, 2012), pancreas (Dumortier *et al.*, 2014), adipose tissue (Jousse *et al.*, 2014) and skeletal muscle (Da Silva Aragao *et al.*, 2014). In this context, it has been revealed that altered protein and gene expression patterns in skeletal muscle and liver caused by poor perinatal nutritional conditions and their persistence through adult life might be an underlying mechanism important, which predisposes to the late emergence of metabolic diseases (Orozco-Solis *et al.*, 2011).

Skeletal muscle plays a critical role in the regulation of glucose homeostasis and lipid utilization coordinated by complex mechanisms (Leandro, Fidalgo, *et al.*, 2012; Kristensen *et al.*, 2014). The understanding of relationship between carbohydrate and fatty acid metabolism in skeletal muscle might be an important pathway for better elucidate the metabolic alterations in the non-communicable disease. Interestingly, da Silva Aragão and colleagues (2014) demonstrated that early protein restriction alters mitochondrial function and glycolytic-oxidative capacity in skeletal muscle of protein-restricted offspring at 150-d-old, verified through the reduced hexokinase activity associated with increased CPT1 and PGC1 expression in skeletal muscle (Ozanne *et al.*, 2005; Jensen *et al.*, 2008; Muhlhausler *et al.*, 2009; Da Silva Aragao *et al.*, 2014).

The liver governs body energy metabolism, actuating as a pivot connecting to various tissues, including skeletal muscle and adipose tissue (Rui, 2014). The liver plays a key role in

maintaining glucose homeostasis through the gluconeogenesis and glycogenosis process and it was shown that increased gluconeogenesis possibly contributes to later outcome as hyperglycaemia and insulin resistance in protein-restricted rats (Kwong *et al.*, 2007; Jia *et al.*, 2012). However, the short and long-term effects of maternal protein-restriction on the phenotypic profile of skeletal muscle and liver remain to be fully evidenced.

In this context, we hypothesized that early protein restriction produces specific metabolic alteration on different muscle and liver, which might be linked to metabolic disorders in later life. To test this hypothesis, here we examined the short- and long-term effects of maternal protein restriction on metabolic properties of two skeletal muscle and liver in male rats offspring.

Methods

The experimental protocol was approved by the Ethical Committee of the Biological Sciences Centre (protocol 23076 019345/2013-81), Federal University of Pernambuco, Brazil. All efforts were made to minimize animal discomfort and the number of animals used; in addition, we followed the Guidelines for the Care and Use of Laboratory Animals.

Animals and experimental groups

Virgin female albino Wistar rats (Rattus novergicus) were obtained from the Academic Centre of Vitoria de Santo Antão (CAV), Federal University of Pernambuco, Brazil. Animals were maintained at room temperature of 22 ± 1°C with controlled light–dark cycle (dark 18:00–06:00 hours). Standard laboratory chow (52% carbohydrate, 21% protein, and 4% lipids - Labina, Purina Agriband, São Paulo, Brazil) *ad libitum* up to the 3-month, when rats were mated (2 females for 1 male). The day on which spermatozoa were identified in vaginal smear was considered as the conception and the pregnant rats were transferred to individual cages: mothers fed with 17% protein diet (n=5, normal protein group, Np) and mothers fed with 8% casein diet (low protein group, Lp, n=5) and water ad libitum. Both diets were isoenergetic (**Table 1**) and were offered during pregnancy and lactation. Diets were elaborated at the Laboratory of Experimental Nutrition-CAV, Federal University of Pernambuco, according to the American Institute of Nutrition – AIN-93 (Reeves *et al.*, 1993). The casein was previously analysed and showed 85% of purity (85g of protein for each 100g of casein).

During the suckling period, the offspring were maintained as litters of eight pups randomly. At weaning, three or four male offspring of each litter were randomly housed in collective

cages and received a standard diet and water ad libitum. The experimental groups were formed with one or two or rats from each mother. All experiments analyses were performed in male offspring at 30-d-old and 90-d-old.

Liver and skeletal muscle sampling

Separate groups of Np (n = 5) and Lp (n = 5) rats were killed by decapitation and their liver, soleus and EDL muscles were collected. The soleus and EDL muscles and liver were carefully dissected and frozen in liquid nitrogen and kept at – 80°C until assayed for gene and protein expression. All rats were euthanized between 02:00-05:00 p.m under fasting condition of 4-5 hours.

RNA extraction, reverse transcription and quantitative PCR (qPCR).

Total RNA was extracted from muscle tissues with Tripure reagent (Roche, Meylan, France) according to the manufacturer's instructions. 10µL of Tripure reagent were added per mg of tissue powder. Briefly, 10µL of Trizol were added per mg of tissue and the resulting suspension was homogenized using a Precellys Lysing kit (Bertin, Montigny-le-Bretonneux, France) according to the manufacturer's instructions. After grinding, 1/4 volume of chloroform was added, the preparation vortexed 3 x 15 s, incubated at room temperature for 5 min and centrifuged for 15 min at 15,000 g at 4°C.

RNA was precipitated by addition of 1/2 volume of isopropanol (Carlo Erba, Val-de-Reuil, France) and centrifugation (15 min at 15,000g at 4°C). RNA-containing pellets were washed sequentially with 70% and 95% ethanol (Carlo Erba), dried, and dissolved in 100 µl RNase-free water. The supernatant containing Trizol was used for proteins extraction.

Reverse transcription was performed using an RT-TAKARA kit (Primescript TM, Dalian, Japan) using 1µg of RNA as template and following the manufacturer's instructions. Briefly, samples were heated for 10 min at 65 °C. 4µL PrimeScript Buffer 5x, 1µl oligodT (50 µM), 4µl random hexamers and 1µl of PrimeScript RT Enzyme Mix, were sequentially added, followed by a 15 min incubation at 37 °C and 15 s at 85 °C. RNA was removed by incubation with 1µL of RNase H for 20 minutes at 37 °C. Reverse transcription reactions were brought to 200 µl final volume by adding RNase free water, and stored at -20°C. Real-time quantitative PCR amplification (qPCR) was performed using a Rotor-Gene Real-Time PCR System (Labgene Scientific Instruments, Archamps, France). Sequences of primers used in this study are reported in **Table 2**.

Reactions were incubated at 95°C for 10 min, followed by 40 cycles of denaturation (95°C, 10 s), annealing (58-62 °C depending on the primer sets, 30 s) and elongation (72 °C, 30 s). mRNA expression levels of hexokinase 2 (HK2), phosphofructokinase (PFK), pyruvate dehydrogenase kinase 4 (PDK4), citrate synthase (CS), Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC1α), Carnitine palmitoyltransferase I (CPT1) and beta hydroxyacyl-coenzyme a dehydrogenase (βHAD) were performed in soleus and EDL muscle. In addition, mRNA expression levels of glucose 6 phosphatase (G6Pase), phosphoenolpyruvate kinase (PEPCK), pyruvate kinase (PKL), fatty acid synthase (FAS), CS, βHAD and PGC1α were analysed in liver. All results are represented as arbitrary units (A.U.) derived from a standard calibration curve derived from a reference sample. A PCR for each sample was carried out in duplicate for all cDNAs and for the ribosomal protein L19 control (RPL19). As a further control, qPCR amplicons were analyzed by agarose gel.

Proteins extractions and Western Blotting procedures

Supernatants containing Trizol it was precipitated with isopropanol for protein extraction. Pellets generated from protein lysates were incubated 15 minutes and washed with guanidine hydrochloride 0.3M in ethanol 95%. After centrifugation (3000g, 5 min), protein were solubilized in sodium dodecyl sulfate (SDS1%). Soluble proteins concentrations was determined using the Bradford method. Bovine serum albumin was used as a standard and optical densities were read at 595 nm on a microplate plate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, MA).

Protein samples were adjusted to a final concentration of 10 mg/µl. After addition of Laemmli sample buffer (150 mM Tris HCl, 36% glycerol, 3% SDS, 12% β-mercaptoethanol, 0.03 % bromophenol blue) and denaturation, proteins were separated by standard SDS-PAGE as described Pirola (Pirola *et al.*, 2003). Protein size markers (Precision Plus Protein Standards, Biorad) were deposited in parallel.

Separated proteins were then transferred to PVDF membranes by semi-dry blotting using a Biorad Transblot Turbo Blotting apparatus. The following primary antibodies were used: anti HK2 (sc-6521, Santa Cruz biotechnologies), anti PFK (sc-67028, Santa Cruz Biotechnologies), anti CS (sc-242444, Santa Cruz Biotechnologies), anti PGC1α (sc-8655, Santa Cruz Biotechnologies), anti β|HAD (sc-292196, Santa Cruz Biotechnologies), anti CPT1 (sc-20670, Santa Cruz Biotechnologies), anti PDK4 (sc-14495, Santa Cruz Biotechnologies) Anti-mouse, rabbit or goat secondary antibodies were used as appropriate, and revelation was made using the ECL reagent Lumina Forte (Merk Millipore, Darmstadt,

Germany) for 2 minutes. Chemiluminescence was acquired on a ChemiDocTM XRS+ camera using the Image Lab 4.1 software (Biorad).

Statistical analysis

Experimental results are expressed as means ± SEM. Statistical analysis between Np and Lp groups were assessed by two-way ANOVA followed by Bonferroni's test. The significance level was fixed to $P < 0.05$. Statistical analysis was performed by using GraphPad Prism 5.0 software.

Results

In soleus of 30 d-old rats, mRNA expression of Hk2, PFKm CPT1β, βHAD, PGC1β and Cs were similar between groups (**Fig. 1**), but βHAD (Np:1.3±0.04 vs. Lp:0.97±0.09, $p<0.05$) and PDK4 (Np:1.52±0.13 vs. Lp:0.92±0.16, $p<0.05$) were downregulated in soleus of Lp rats (**Fig.1**). In addition, protein expression of PDK4 (Np: 0.97±0.003 vs. Lp: 0.75±0.004, $p<0.05$) and βHAD (Np: 0.054±0.002 vs. Lp: 0.04±0.005, $p=0.07$) also were down-expressed in soleus of Lp group (**Fig. 1**). Interestingly, protein expression of HK2 (Np: 0.10±0.006 vs. Lp: 0.16±0.006, $p<0.05$) was upregulated in Lp group in comparison to Np group (**Fig.1**).

The transcriptional profile of EDL was also affected by early protein restriction. It was observed that PDK4, CPT1β and βHAD mRNA expression was similar between groups (**Fig. 2**). However, Hk2 (Np:0.53±0.07 vs. Lp: 0.35±0.06, $p<0.05$), PFK (Np:0.88±0.07 vs. Lp:0.49±0.08, $p<0.05$), Cs (Np:1.13±0.09 vs. Lp:0.77±0.05, $p<0.05$) and PGC1α (Np:0.66±0.05 vs. Lp:0.41±0.03, $p<0.05$) expression was downregulated in Lp rats (**Fig. 2**). In addition, protein expression of HK2 (Np: 1.39±0.16 vs. Lp: 0.85±0.12, $p<0.05$) and PFK (Np: 0.68±0.05 vs. Lp: 0.51±0.05, $p=0.06$) also were down expressed in EDL from Lp rats (**Fig. 2**), but no differences were seen in protein expression of βHAD and CS (**Fig. 2**).

In the liver at 30d-old, G6Pase (Np:0.38±0.06 vs. Lp:0.74±0.1, $p<0.05$), Cs (Np:1.10±0.1 vs. Lp:1.5±0.07, $p<0.05$) and βHAD (Np:0.46±0.06 vs. Lp:0.68±0.07, $p<0.05$) were upregulated in Lp rats (**Fig. 3**). However, no differences between Np and Lp rats were observed in the transcript levels of the PEPCK, FAS and PGC1α (**Fig. 3**). In addition, protein expression of βHAD and PGC1α were similar between Np and Lp conditions.

Male rats offspring at 90 d-old were used to determine the long-term effects of maternal protein restriction on metabolic profile in skeletal muscle and liver. In soleus 90 d-old, mRNA expression of PFKm CPT1β, βHAD, CS and PGC1β were similar between groups (**Fig. 4**), but HK2 (Np:3.42±0.56 vs. Lp:2.08±0.23, $p=0.05$) and PDK4 (Np:3.33±0.23

vs. Lp:2.47±0.24, $p<0.05$) were downregulated in soleus of Lp rats (**Fig.4**). However, there were no differences in the protein expression of HK2, PDK4 and βHAD between the groups (**Fig. 4**).

In EDL muscle, no differences between Np and Lp group were observed in mRNA and protein expression of Hk2, PFK, and βHAD (**Fig. 5**). In addition, no differences were found in mRNA expression of CPT1β, CS and PGC1α (**Fig. 5**). However, we observed that transcript levels of gene PDK4 (Np: 3.27±0.13 vs. Lp: 1.52±0.35, $p<0.05$) was downregulated in Lp rats when compared to Np group (**Fig. 5**).

In the liver at 90d-old, gene expression of PEPCK, G6Pase, βHAD and CS were similar between Np and Lp condition (**Fig. 6**). Interestingly, mRNA expression of FAS (Np: 0.35±0.04 vs. Lp: 0.61±0.07, $p<0.05$) was upregulated in the Lp rats (**Fig. 6**). Moreover, mRNA of PGC1α (Np: 0.54±0.07 vs. Lp: 0.33±0.05, $p<0.05$) was downregulated in liver, but protein expression of PGC1α it was no changed between Np and Lp groups (**Fig. 6**). Lastly, we observed that protein expression of βHAD (Np: 0.7±0.08 vs. Lp: 0.29±0.05, $p<0.05$) was upregulated in Lp rats when compared to Np group.

Discussion

In the present study, we evaluated the short and long-term effects of a perinatal low-protein diet on the expression of genes for key-proteins of the glucose and fatty acid cycle in the two different types of skeletal muscle (soleus, typically oxidative; and EDL, typically glycolytic) and in the liver. Clearly, our findings showed that maternal protein restriction alters in the short and long-term the metabolic gene properties of offspring skeletal muscle and liver in type-age-specific manner.

Previously, it was shown that mice subjected to protein restriction during gestation exhibit pronounced effects on gene expression in skeletal muscle and liver (Mortensen *et al.*, 2010). In particular, mitochondrial genes associated with oxidative phosphorylation were upregulated in liver and downregulated in skeletal muscle of protein-restricted rats (Mortensen *et al.*, 2010).

On the other hand, previous reports have revealed that gene expression dysregulation of proteins involved in the glucose- and fatty acid metabolism can impair the insulin-signaling pathway, leading to insulin resistance, obesity and cardiovascular disease (Hirabara *et al.*, 2006). Although a relation between maternal protein restriction during pregnancy and lactation and the development of non-communicable disease in offspring has been previously described, its underlying mechanisms are poorly understood. Altogether, our data bring new

insights into the etiologic mechanisms underlying the genes dysregulation in protein-restricted rats, which can help to elucidate the development of insulin resistance, obesity and cardiovascular disease in individuals submitted to protein restriction during gestation and/or lactation.

HK2 and PFK represent two important genes that are involved in glucose transport and glycolysis in skeletal muscle (Jones e Bianchi, 2015). Previously, da Silva Aragão e Colleagues (2014), observed that enzymatic activity of HK2 it was downregulated in soleus but no in EDL muscle from adult offspring subjected to protein restriction during gestation and lactation (Da Silva Aragao *et al.*, 2014). Similarly, our data showed that gene expression of HK2 it is downregulated in soleus of adult protein-restricted offspring without modification in EDL muscle. In addition, analyzing glycolytic enzymes in EDL muscle at 30d, we found that mRNA and protein expression of HK2 and PFK it was downregulated in juvenile offspring of protein-restricted rats. Suggesting that rate of glycolysis and use of glucose as fuel energy might be impaired in skeletal muscle of protein-restricted rats.

Previous reports have shown that accumulation of fatty acids into the mitochondria it is associated with higher susceptibility to development of insulin resistance and obesity (Schrauwen *et al.*, 2010; Jun *et al.*, 2011). In this way, we did not detect any difference in gene and protein expression levels of CPT1 between control and Lp rats. Suggesting that it drives the transport of long-chain acids into mitochondria are similar in skeletal muscle of rats subjected or no to protein restriction during gestation and lactation. However, at short-term we found an attenuation of mRNA expression of βHAD, leading to the idea that lipid oxidation could be impaired in oxidative muscle of protein-restricted pups.

In addition, impaired mitochondrial oxidative metabolism might leads to defective insulin signaling and insulin resistance (Kristensen *et al.*, 2014). In this way, it was observed that PGC1α it is downregulated in skeletal muscle of patients with inherited insulin resistance (Kristensen *et al.*, 2014). Accordingly, our data showed that PGC1a could be also downexpressed in skeletal muscle and liver of protein-restricted rats.

Pyruvate dehydrogenase (complex PDC) exhibit an important function to glucose homoeostasis, allowing the pyruvate to be channeled to the TCA cycle for complete oxidation (Jeoung *et al.*, 2006). In the starved state, PDC complex is inactivated as a consequence of phosphorylation of its dehydrogenase component through increases of PDK4, which helps maintain glucose levels by conserving substrates for gluconeogenesis pathways (Jeoung *et al.*, 2006).

Interestingly, our data demonstrated that PDK4 gene expression is downregulated at short- and long-terms in soleus and EDL skeletal muscle of protein-restricted rats. These results suggest that during starved state, skeletal muscle from protein-restricted rats possibly exhibit enhanced PDC complex activity.

During fasting, pyruvate released from skeletal muscle through of glycogenolysis and glycolysis or glycerol released from adipose tissue through lipolysis can be utilized by hepatocyte to produce glucose through gluconeogenesis (Jeoung *et al.*, 2006; Rui, 2014). The rate of gluconeogenesis is determined by availability of gluconeogenic substrates and the expression of gluconeogenic enzymes PEPCK and G6Pase, which control key steps of gluconeogenesis (Rui, 2014).

It has been shown previously that maternal protein restriction (9% casein) during gestation induced an increase in hepatic gene expression of PEPCK in male fetus, but no in females (Kwong *et al.*, 2007). However, analyzing protein-restricted rats at 30-d and 90d-old, we did not observe any difference in hepatic mRNA expression of PEPCK in male offspring. These differences might be explained by the severity and timing of the nutritional insult and the age of the rats at which the molecular analysis was performed.

On the other hand, it was observed that fetus rats and piglets from dams subjected to protein restriction during gestation, exhibited enhanced hepatic G6Pase expression, which it was accompanied by hypomethylation and more histone H3 acetylated on the G6Pase promoter (Franko *et al.*, 2009; Jia *et al.*, 2012). Suggesting that epigenetic pathways could be involved in the hepatic gluconeogenic alterations in protein-restricted animals.

Interestingly, our data showed that mRNA expression of G6Pase it was enhanced in juvenile rats (30-d-old) but without any modification in the adulthood of rats subjected to protein restriction during gestation and lactation. Therefore, because the G6Pase it is upregulated at 30d-old in protein-restricted rats, we theorize that hepatic gluconeogenic pathway might be important for long-term consequences on glucose homeostasis. However, this hypothesis still requires further experimental verification.

Hepatic genes involved in the lipogenic pathways, as fatty acid synthase (FAS) and β-oxidation (βHAD) are important in the regulation of hepatic lipid metabolism (Jensen-Urstad e Semenkovich, 2012; Nascimento *et al.*, 2013). Interestingly, clinical and experimental studies have identified a correlation between hepatic lipid content and the development of insulin resistance in liver (Savage *et al.*, 2006; Kumashiro *et al.*, 2011).

Notably, our observation showed that βHAD and Cs were upregulated in protein-restricted rats at 30-d-old. These results indicate that possibly the fatty acid oxidation or

increased production of ATP it is enhanced in liver of Lp rats. On the other hand, PGC-1α is an important transcription factor involved in the regulation of mitochondrial biogenesis and whole cellular energy status (Scarpulla *et al.*, 2012). Lukaszuk and cols (2015) demonstrated that a modest PGC-1α protein ablation resulted impaired fatty acid oxidation, evidenced by diminished β-HAD expression (Lukaszuk *et al.*, 2015).

In conclusion, the present study suggests that maternal protein restriction alters in the short and long-term the gene expression of proteins involved in the glucose- and fatty acid metabolism in skeletal muscle and liver. These persistent alterations might contribute to the development of metabolic dysfunctions later in life.

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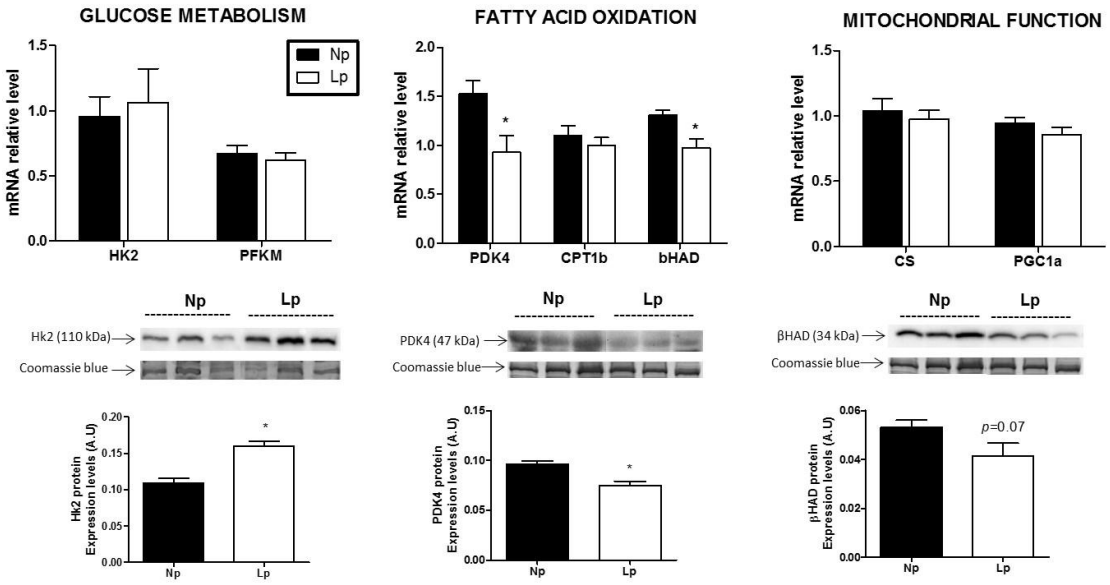

Figure 1. Evaluation of mRNA and protein expression levels of metabolic genes regulating energy metabolism and mitochondrial function in soleus muscle from rats at 30d-old born to normal (black bars, Np group, n=5) or from protein-restricted (white bars, Lp group, n=5) dams during gestation and lactation. All pups were fed a standard chow diet at weaning. Values are means ±SEM.*Mean values were significantly different from those of the Np group

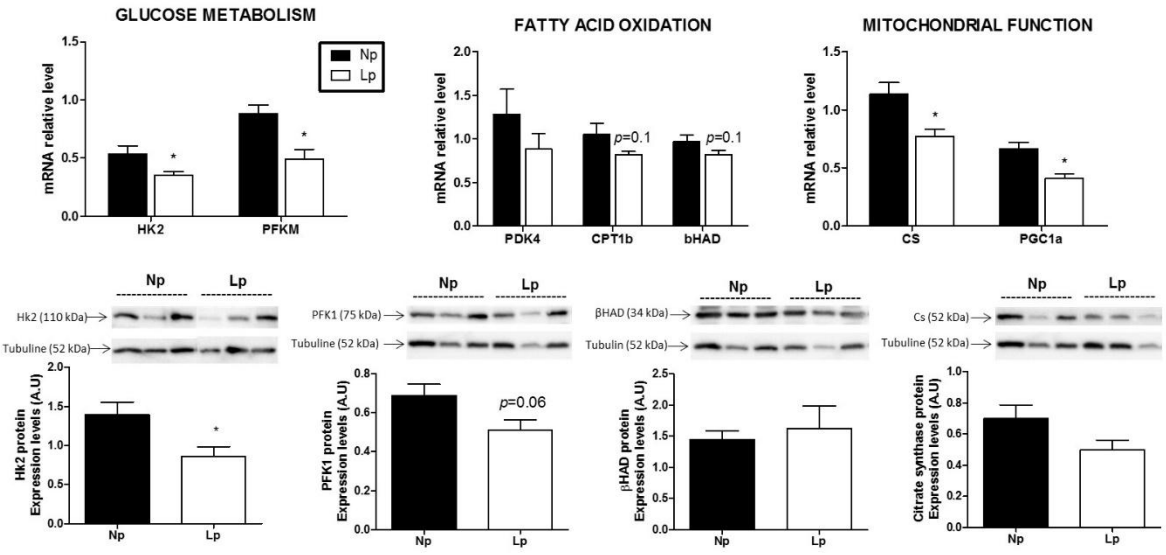

Figure 2. Evaluation of mRNA and protein expression levels of metabolic genes regulating energy metabolism and mitochondrial function in EDL muscle from rats at 30d-old born to normal (black bars, Np group, n=5) or from protein-restricted (white bars, Lp group, n=5) dams during gestation and lactation. All pups were fed a standard chow diet at weaning. Values are means ±SEM.*Mean values were significantly different from those of the Np group

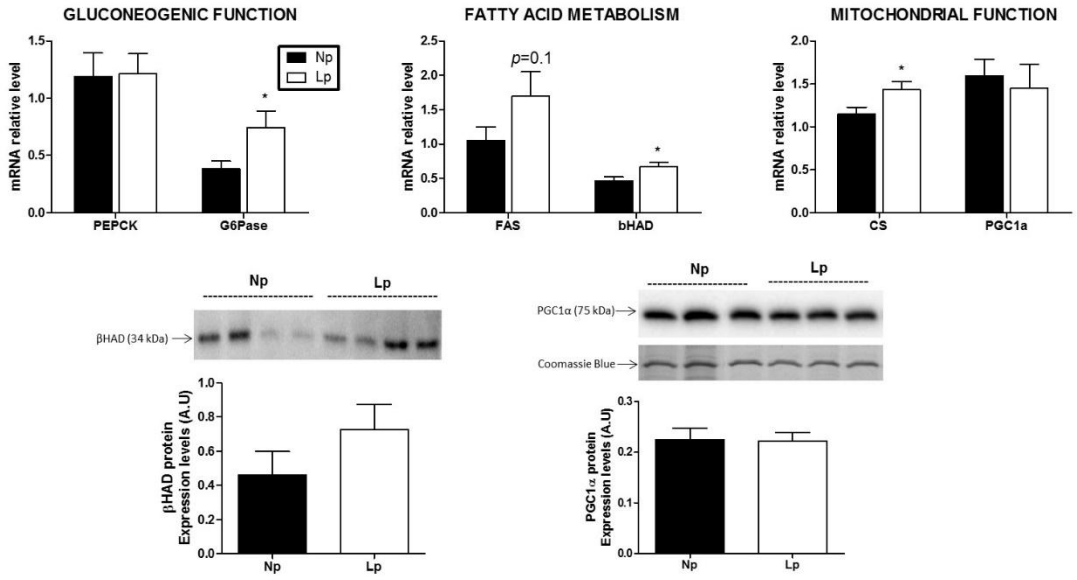

Figure 3. Evaluation of mRNA and protein expression levels of metabolic genes regulating energy metabolism and mitochondrial function in liver from rats at 30d-old born to normal (black bars, Np group, n=5) or from protein-restricted (white bars, Lp group, n=5) dams during gestation and lactation. All pups were fed a standard chow diet at weaning. Values are means ±SEM.*Mean values were significantly different from those of the Np group

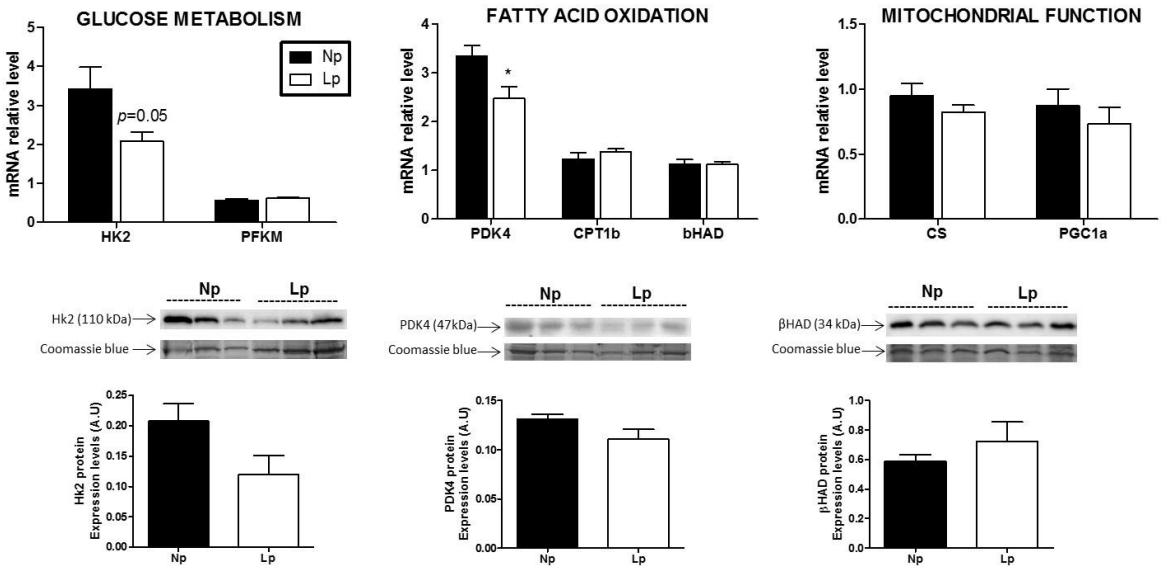

Figure 4. Evaluation of mRNA and protein expression levels of metabolic genes regulating energy metabolism and mitochondrial function in soleus muscle from rats at 90d-old born to normal (black bars, Np group, n=5) or from protein-restricted (white bars, Lp group, n=5) dams during gestation and lactation. All pups were fed a standard chow diet at weaning. Values are means ±SEM.*Mean values were significantly different from those of the Np group

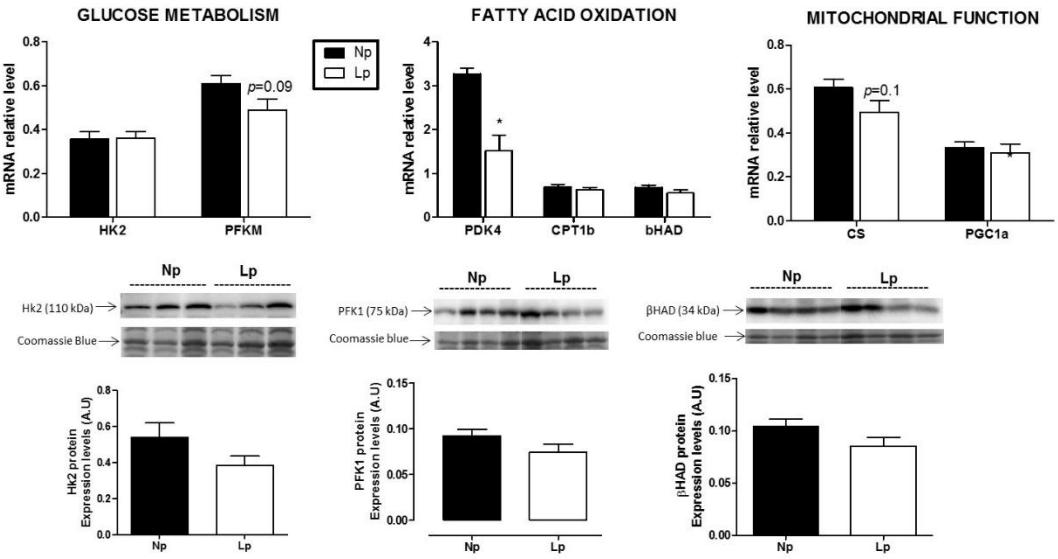

Figure 5. Evaluation of mRNA and protein expression levels of metabolic genes regulating energy metabolism and mitochondrial function in EDL muscle from rats at 90d-old born to normal (black bars, Np group, n=5) or from protein-restricted (white bars, Lp group, n=5) dams during gestation and lactation. All pups were fed a standard chow diet at weaning. Values are means ±SEM.*Mean values were significantly different from those of the Np group

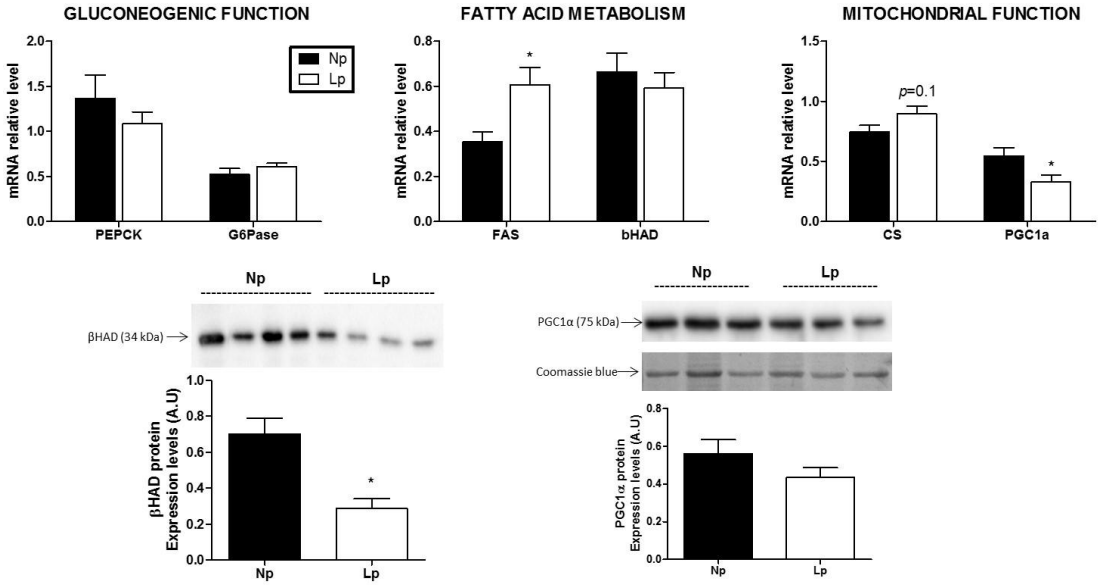

Figure 6. Evaluation of mRNA and protein expression levels of metabolic genes regulating energy metabolism and mitochondrial function in liver from rats at 90d-old born to normal (black bars, Np group, n=5) or from protein-restricted (white bars, Lp group, n=5) dams during gestation and lactation. All pups were fed a standard chow diet at weaning. Values are means ±SEM.*Mean values were significantly different from those of the Np group

Table 1. Nutritional Composition of the experimental diets (g/100g diet)

Nutrient	Normal protein (17% protein)	Low protein (8% protein)
Casein (85%)*	20	9.41
Dextrin cornstarch	13	13.2
Cellulose	5	5
Sucrose	10	10
Cornstarch	39.74	50.34
Soybean oil	7	7
Choline	0.25	0,25
Methionine	0.3	0.3
Vitamin mix	1	1
Mineral mix	3.5	3.5
Energy density (kJ/g)	16.26	16.26

* The casein used in preparation of diet was 85% purity.

Table 2. Sequences of primers used for the real-time RT-PCR analysis.

Gene	F/R	Tm (°C)	Sequence 5' - 3'	Gene Bank
Hk2	F	60	CTTCTCGTTCCCCTGCCACC	NM_012735
	R		CCATGTAGCAGGCGTTGCTG	
PFKM	F	60	GACGTGACCAAGGCTATGGA	
	R		CAGTCCAGCCTCCAACATAG	
PDK4	F	58	AGTGTGCAAAGATGCTCTGC	
	R		AGAGCATGTGGTGAAGGTGTG	
CPT1β	F	60	AGGCAGTAGCTTTCCAGTTCAC	NM_013200
	R		CACACCCCTAAGGATACCATTC	
βHAD	F	58	GGGAAGGTCATCATTGTGG	NM_130826
	R		ACTCCTTCCTGGAGGATTC	
CS	F	58	ACTACATCTGGAACACACTC	NM_130755
	R		TCATGCCATAGTACTGGAGC	
PGC1α	F	60	TCCTCTGACCCCAGAGTCAC	
	R		CTTGGTTGGCTTTATGAGGAGG	
PEPCK	F		----------	
	R		----------	
G6Pase	F		----------	
	R		----------	
FAS	F		----------	
	R		----------	
RPL19	F	58	CTGAAGGTCAAAGGGAATGTG	NM_001159483
	R		GGACAGAGTCTTGATGATCTC	

F: Foward sequence; R: Reverse sequence

Sequences of hexokinase 2 (HK2), Phosphofructokinase (PFKM), pyruvate dehydrogenase kinase 4 (PDK4), citrate synthase (CS), Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC1α), Carnitine palmitoyltransferase I (CPT1), beta hydroxyacyl-coenzyme a dehydrogenase (βHAD), glucose 6 phosphatase (G6Pase), phosphoenolpyruvate kinase (PEPCK), pyruvate kinase (PKL), fatty acid synthase (FAS) and ribosomal protein L19 (RPL19).

7. Conclusões e Perspectivas

Ratos expostos à restrição proteica durante a gestação e lactação apresentam antes do aumento de pressão arterial:

- Aumento de atividade simpática e respiratória;
- Maior resposta simpatoexcitatória e ventilatória durante ativação de quimiorreceptores periféricos;
- Maior resposta ventilatória durante ativação de quimiorreceptores centrais.

Essas observações mostram que uma hiperatividade simpático-respiratória associada com uma maior quimiossensibilidade central e periférica podem contribuir para o desenvolvimento de hipertensão arterial em ratos expostos à restrição proteica perinatal.

Aos 90 dias de vida, ratos que foram expostos à restrição proteica perinatal apresentaram:

- Aumento de pressão arterial;
- Déficits na expressão gênica e na atividade de enzimas antioxidantes no bulbo;
- Expressão gênica semelhantes de receptores glutamatérgicos no bulbo.

A remoção de quimiorreceptores periféricos no início da vida foi capaz de atenuar a pressão arterial de ratos expostos à restrição proteica perinatal na vida adulta.

Ratos expostos a restrição proteica perinatal exibiram a curto (30 dias) e longo prazo (90 dias), importantes modificações na expressão gênica e proteica de enzimas envolvidas no metabolismo de ácidos graxos e glicose no músculo esquelético e no fígado.

Futuros trabalhos serão conduzidos para melhor compreender os mecanismos pelos quimiorreceptores periféricos podem contribuir para o desenvolvimento de hipertensão arterial em ratos expostos à restrição proteica materna. Adicionalmente, investigações acerca da via glicose-ácido graxo serão realizadas no tecido adiposo para uma melhor abordagem gênica e metabólica nesses animais.

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