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Impact of taurine supplementation on blood pressure in gestational protein-restricted offspring: Effect on the medial solitary tract nucleus cell numbers, angiotensin receptors, and renal sodium handling

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Abstract

Objective: The current study considers changes of the postnatal brainstem cell number and angiotensin receptors by maternal protein restriction (LP) and LP taurine supplementation (LPT), and its impact on arterial hypertension development in adult life.

Methods and results: The brain tissue studies were performed by immunoblotting, immunohistochemistry, and isotropic fractionator analysis. The current study shows that elevated blood pressure associated with decreased fractional urinary sodium excretion (FE_{Na}) in adult LP offspring was reverted by diet taurine supplementation. Also, that 12-day-old LP pups present a reduction of 21% of brainstem neuron counts, and, immunohistochemistry demonstrates a decreased expression of type 1 angiotensin II receptors (AT_1R) in the entire medial solitary tract nuclei (nTS) of 16-week-old LP rats compared to age-matched NP and LPT offspring. Conversely, the immunostained type 2 AngII (AT_2R) receptors in 16-week-old LP nTS were unchanged.

Conclusion: The present investigation shows a decreased FE_{Na} that occurs despite unchanged creatinine clearance. It is plausible to hypothesize an association of decreased postnatal nTS cell number, AT_1R/AT_2R ratio and FE_{Na} with the higher blood pressure levels found in taurine-deficient progeny (LP) compared with age-matched NP and LPT offspring.

Keywords

Arterial hypertension, low-protein diet, taurine, angiotensin receptors, solitary tract nucleus, kidney function, natriuresis

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Introduction

The concept of fetal programming suggests that the fetus is programmed in uteri to develop a number of adult diseases, including arterial hypertension and diabetes mellitus.[1] Intrauterine growth restriction (IUGR) has been associated with maternal low protein intake and, although the specific nature of this insult is unclear, a number of mechanisms have been proposed. In uteri programming of hypertension via alteration of the renin angiotensin system (RAS) before birth has attracted great attention. Since these initial discoveries, most intrinsic components of the RAS, including angiotensinogen, angiotensin, and converting enzymes, have been well described and demonstrated in different areas of the central nervous system (CNS).[2,3] Notably, most previous studies in this field have focused on the central and peripheral angiotensin receptors and their roles in prenatal imprinting. The challenge for all of us is to understand in depth the mechanisms by which antenatal stress may alter normal neurophysiology of the brain RAS. Intrauterine

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malnutrition has been known to induce alterations in the development of several tissues, among which are the CNS.[3] The role of the CNS in the control of blood pressure and hydrosaline homeostasis has been demonstrated by several studies.[4,5] Also, the central role of the RAS in the control of blood pressure and hydroelectrolyte homeostasis has been widely demonstrated.[6,7] The medial solitary tract nucleus (nTS), the central site of termination of baroreceptive afferents, is intimately involved in arterial pressure control. This nucleus contains a high density of angiotensin II (AngII) AT_1 receptors (AT_1R) located both presynaptically, on vagal and carotid sinus afferents, and on interneurons.[8] However, in the IUGR models the cytology pattern of medial solitary tract nuclei (nTS) modulation of AngII receptors is not well known. AngII lowers blood pressure and heart rate after injection of low doses in the nTS, as previously reported in several strains of rats.[9,10] This response can be blocked completely by the nTS injection of an AT_1R antagonist.[11] The current study pays attention to changes of the postnatal nTS angiotensin receptors by maternal protein restriction (LP), and its impact on in uteri programming of hypertension in adult life. Taking in account the above findings, the purpose of the present study, firstly, was to determine whether maternal protein restriction during whole pregnancy alters the nTS cytological pattern and expression of AT_1R and AT_2R in 16-week-old (LP) offspring; these data were compared with those of age-matched appropriate normal-protein ingestion (NP) controls. On the other hand, a study has also shown previously that the level of taurine (2β-amino ethanesulphonic acid) is markedly reduced in the plasma of fetuses of dams fed a low-protein diet.[12] Maternal taurine supplementation of the LP diet restored to normal the taurine levels in fetal plasma.[13] Thus, the second aim of this study was to determine if taurine, added to LP content chow (LPT) of these dams, could provide prevention against hypertension development when compared to LP offspring. We also hypothesized that arterial hypertension in adult life may result, at least in part, from nTS disorders in association with modified urinary sodium handling, evaluated by lithium clearance, in conscious maternal LP intake rats, when compared with their appropriate experimental controls (NP and LPT groups).

Materials and methods

Animals

The experiments were conducted on age-matched, female offspring of sibling-mated Wistar rats (0.250–0.300 kg) allowed free access to water and normal rat chow. The general guidelines established by the Brazilian College of Animal Experimentation (Protocol #2575-1) were followed throughout the investigation. Our local colonies originated from a breeding stock supplied by the University of Campinas Animal Breeding Center, Campinas, SP, Brazil. Immediately after weaning at 3 weeks of age, animals were maintained under controlled temperature (25°C) and lighting conditions (0700 h–1900 h), with free access to tap water and standard rodent laboratory chow (Nuvital, Curitiba, PR, Brazil with Na^+ content: 135 ± 3 μEq/g; K^+ content: 293 ± 5 μEq/g) and followed up to 12 weeks of age. Animals were mated and the day that sperm were seen in the vaginal smear was designated as day one of pregnancy. The dams were maintained on isocaloric standard rodent laboratory (with normal protein content (NP), 17% protein), low protein content (LP) (6% protein) or 2.5% taurine added to the LP content chow (LPT) ad libitum intake throughout the entire pregnancy. The maternal body weight gain for all groups was determined between the first and third gestational period. All groups resumed the NP chow intake after delivery. The male pups were followed and maintained with normal chow until adulthood. The offspring food consumption (subsequently normalized for body weight) and body weight were determined every day, all over 16 weeks. The data relating to body weight were obtained on a daily basis. However, in this study data were presented as the weekly average of all daily weights.

Blood pressure measurement

Systolic blood pressure was measured in conscious 6-, 8-, 10-, 12-, 14- and 16-week-old rats (LP, LPT and NP; $n = 12$ for each group) by an indirect tail-cuff method using an electrosphygmomanometer (IITC Life Science – BpMonWin Monitor Version 1.33) combined with a pneumatic pulse transducer/amplifier. This indirect approach allowed repeated measurements with a close correlation (correlation coefficient = 0.975), compared to direct intra-arterial recording.[14–17] The mean of three consecutive readings represented the blood pressure.

Total cell and neuron quantification of the medulla oblongata

The cell and neuron quantification followed the technique described by Herculano-Houzel and Lent (2005). Briefly, five 12-day-old and adult offspring from different mothers of NP, LP, and LPT rats were sacrificed and perfused transcardially with saline, followed by 4% buffered paraformaldehyde. The medulla oblongata was removed from the brain using the foramen magnum as the inferior limit of anatomical landmarks for dissection. A suspension of nuclei is obtained through mechanical dissociation of the fixed brain tissues in a standard solution (40 mM sodium citrate and 1% Triton X-100), using a 40-ml glass Tenbroeck tissue homogenizer. Using at least 1 ml of dissociation solution per 100 mg of brain tissue and grinding until the smallest visible fragments are dissolved achieves complete homogenization. The homogenate is collected with a Pasteur pipette and transferred to 15-ml centrifuge tubes. The grinding pestle and

tube are washed several times with dissociation solution and centrifuged for 10 min at 4000 g. Pelleted nuclei are then suspended in phosphate-buffered saline (PBS) containing 1% 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR, USA), to make all of the nuclei visible under ultraviolet illumination. After sufficient agitation, 5-µl aliquots are removed for determination of nuclei density in a hemocytometer. DAPI-stained nuclei are counted under a fluorescence microscope at 400× magnification. Once nuclear density in the suspension is determined by averaging over at least eight samples, the total number of cells in the original tissue is estimated by multiplying mean nuclear density by total suspension volume. For estimates of total neuron number, a 200–500-µl aliquot is removed from the nuclear suspension and immunoreacted for NeuN. Nuclei in the aliquot are collected by centrifugation, resuspended in a 0.2-M solution of boric acid, pH 9.0, and heated for one hour at 75°C for epitope retrieval. Subsequently, nuclei are again collected by centrifugation, washed in PBS, and incubated overnight at room temperature with anti-NeuN mouse immunoglobulin G (IgG) (1:300 in PBS; Chemicon, Temecula, CA, USA). After being washed in PBS, nuclei are incubated in cyanine 3-conjugated goat anti-mouse IgG secondary antibody (1:400 in 40% PBS, 10% goat serum, and 50% DAPI; Accurate Chemicals, Westbury, NY, USA) for two hours, collected by centrifugation, washed in PBS, and then suspended in a small volume of PBS for counting under the fluorescence microscope. Total number of nonneuronal nuclei is calculated by subtracting the number of NeuN containing nuclei from the total number of nuclei.[18]

Renal function evaluation

The renal function tests were performed at 8 and 16 weeks of age in unanesthetized, unrestrained NP (n = six), LP (n = six) and LPT (n = six) male rats. Briefly, 14 hours before the renal test, 60 µmol LiCl 100 g^{-1} body weight was given by gavage. After an overnight fast, each animal received a load of tap water by gavage (5% of the body weight), followed by a second load of the same volume, one hour later, and spontaneously voided urine was collected over a 120-minute period into a graduated centrifuge tube. At the end of the experiment, blood samples were drawn through the cardiac puncture in anesthetized rats and urine and plasma samples were collected for analysis.[14–17]

Western blot

Brain tissue extraction and immunoblotting was performed as previously described.[18,19] Briefly, NP, LP and LPT (n = five for each group) rats (16 weeks old), were anesthetized and subjected to craniotomy. Brainstems were obtained and homogenized in freshly prepared ice-cold buffer (1% Triton X-100, 100 mM Tris, pH 7.4, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM ethylenediaminetetraacetic acid

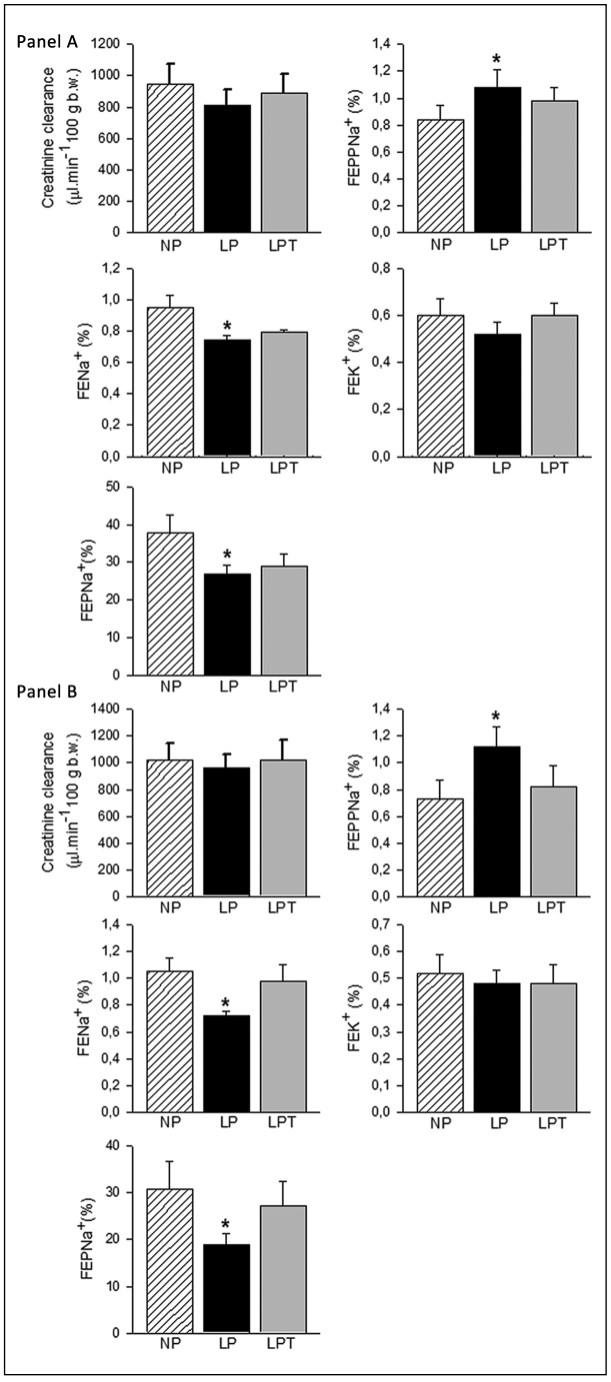

Figure 1. Creatinine clearance (C_{Cr}), fractional sodium excretion (FE_{Na}), proximal (FEP_{Na}) and post-proximal ($FEPP_{Na}$) fractional sodium excretion and fractional potassium excretion (FE_K) in control (NP), maternal protein-restricted (LP) and, LP taurine-supplemented 8-week- (Panel A) and 16-week-old (Panel B) offspring. See Results for statistical analysis details. The data are reported as the means ± SD. *$p \leq 0.05$ vs. NP (analysis of variance (ANOVA); post-hoc Bonferroni's test).

(EDTA), 10 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 0.01 mg aprotinin/ml). Insoluble material was removed by centrifugation (10,000 g) for 25

minutes at 4°C. Protein quantification was performed using the Bradford method. For immunoblotting of total protein extracts, 0.2 mg total protein were suspended in Laemmli sample buffer, boiled for 5 minutes and loaded onto the electrophoresis gel. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electrotransfer and blot followed the same steps as described above for immunoblotting. The nitrocellulose transfers were probed with specific antibodies ($AT1_R$ 1:1000, $AT2_R$ 1:1000). The blots were subsequently incubated in peroxidase-conjugated secondary antibodies (1:10,000). Immunoreactive bands were detected using the enhanced chemiluminescence method (RPN 2108 ECL Western Blotting analysis system; Amersham Biosciences) and detected by pre-flashed Kodak XAR film. Band intensities were quantified by optical densitometry of developed chemiluminescence (Scion Image software, ScionCorp, Frederick, MD, USA). To ensure equal loading, membranes were stained with Coomassie Brilliant Blue dye before blotting. As shown in Figure 1, all membranes were also incubated with β-actin antibody to discard possible inequalities in protein loading and/or transfer. Only homogeneously stained membranes were employed in the study.

Tissue processing, histology and immunohistochemical procedures

Sixteen-week-old male rats from the NP (n = five), LP (n = five), and LPT (n = five) groups were used. The rats were anesthetized with a mixture of ketamine (75 mg.kg^{-1} body weight, intraperitoneally (i.p.)) and xylasine (10 mg.kg^{-1} body weight, i.p.) and monitoring the corneal reflex controlled the level of anesthesia. The animals were then perfused with saline containing heparin (5%) for 15 minutes under constant pressure, followed by perfusion with 0.1 M phosphate buffer (PB; pH 7.4) containing 4% (w/v) paraformaldehyde and 0.1 M sucrose for 25 minutes. After the perfusion, brains were removed and placed in the same fixative for two hours for paraffin embedding. For immunohistochemical analysis we used anti-AT_1R, AT_2R antibodies (Santa Cruz Biotech Inc, CA, USA). Antigen retrieval was performed using 0.01 M citrate buffer (pH 6.0) boiling in a microwave oven (1300 watts) twice for five minutes each. Proteins were immunohistochemically detected using the avidin-biotin-peroxidase method. Briefly, deparaffinized 5-µm-thick sections on poly-l-lysine coated slides were treated with 3% H_2O_2 in PBS for 15 minutes, nonfat milk for 60 minutes, primary antibodies for 60 minutes, and avidin-biotin-peroxidase solution (Vector Laboratories Inc, CA, USA, 1:1:50 dilution). Chromogenic color was accomplished with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich Co., St. Louis, MO, USA) as the substrate to demonstrate the sites of peroxidase binding. The slides were counterstained with Harris's hematoxylin.

Antibodies and chemicals

SDS-PAGE and immunoblotting reagents were obtained from Bio-Rad (Richmond, CA, USA). Hepes, PMSF, aprotinin, dithiothreitol, Triton X-100, Tween 20, glycerol, AngII, and bovine serum albumin (BSA) (fraction V) were from Sigma Chemical Co. (St Louis, MO, USA) and nitrocellulose membranes were from Amersham Corp. (Aylesbury, Bucks, UK). Antibodies against AT_1R, AT_2R, (rabbit polyclonal, AB-1565) for immunoblotting was from Millipore and Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Secondary antibodies and conjugated complexes utilized in immunohistochemistry were from Vector Laboratories Inc (Burlingame, CA, USA). Sodium pentobarbital was from Cristália (São Paulo, Brazil).

Data presentation and statistical analysis

All numerical results are expressed as the mean ± SD of the indicated number of experiments. Plasma and urine sodium, potassium, and lithium concentration were measured by flame photometry (Micronal, B262, São Paulo, Brazil), while the creatinine concentrations and plasma osmolality were determined spectrophotometrically (Instruments Laboratory, Genesys V, USA) and by Wide-range Osmometer (Advanced Inst. Inc, MA, USA), respectively. Creatinine clearance was used to estimate glomerular filtration rate (GFR) and lithium clearance (C_{Li}) was used to assess proximal tubule output.[14–17] Fractional sodium excretion (FE_{Na}) was calculated as $C_{Na}/C_{Cr} \times 100$, where C_{Na} is sodium clearance and C_{Cr} is creatinine clearance. The fractional proximal (FEP_{Na}) and post-proximal ($FEPP_{Na}$) sodium excretion was calculated as $C_{Li}/C_{Cr} \times 100$ and $C_{Na}/C_{Li} \times 100$, respectively. Data obtained over time were analyzed using one-way analysis of variance (ANOVA). Post hoc comparisons between selected means were performed with Bonferroni's contrast test when initial ANOVA indicated statistical differences between experimental groups. Comparisons involving only two means within or between groups were carried out using a Student's t test. The results of blots are presented as direct comparisons of bands in autoradiographs and quantified by densitometry using the Scion Image software (ScionCorp). The level of significance was set at $p \leq 0.05$.

Results

There were no significant differences between plasma sodium, potassium, lithium, osmolality and urine flow (Table 1) in NP rats, compared with the LP group. The maternal body weight for all groups, at first (NP1: 207.8 ± 11.23 g; LP1: 216.2 ± 16.7 g and LPT1: 203.9 ± 15.03 g) and third gestational week (NP3: 265.8 ± 19.42 g; LP3: 255.2 ± 21.82 g and LPT3: 251.2 ± 16.6 g), was similar ($p > 0.05$ for each group). Otherwise, the maternal gestational body weight gain was significantly higher in NP and LPT (NP1 vs. NP3: 59%; LPT1 vs. LPT3: 48%,$p < 0.0001$)

Table 1. Serum sodium, potassium, lithium levels, osmolality, urine flow, and body weight (b.w.) as related to age and blood pressure (BP) in maternal protein-restricted (LP), taurine-supplemented (LPT) and maternal normal-protein intake offspring (NP).

Groups	Na^+ (mM)	K^+ (mM)	Li^+ (μM)	Osmolality ($mOsm.kg^{-1}H_2O$)	V (μl/min/100 g b.w.)	Body weight (g) 16 weeks old	BP (mmHg) 16 weeks old
NP (n = 6)	136 ± 2.8	4.6 ± 0.3	84 ± 21	293 ± 8	51.2 ± 12.5	381 ± 25.5	118.8 ± 8.7
LP (n = 6)	141 ± 4.3	4.2 ± 0.5	81 ± 19	297 ± 7	51.2 ± 6.8	415.4 ± 16	137.9 ± 6.9[a]
LPT (n = 6)	139 ± 3.7	4.3 ± 0.6	83 ± 18	296 ± 9	50.4 ± 7.6	401.8 ± 22.8	123.2 ± 5.6

Data are reported as means ± SD. [a]$p \leq 0.05$ vs. NP (one-way analysis of variance (ANOVA); post-hoc Bonferroni's test).

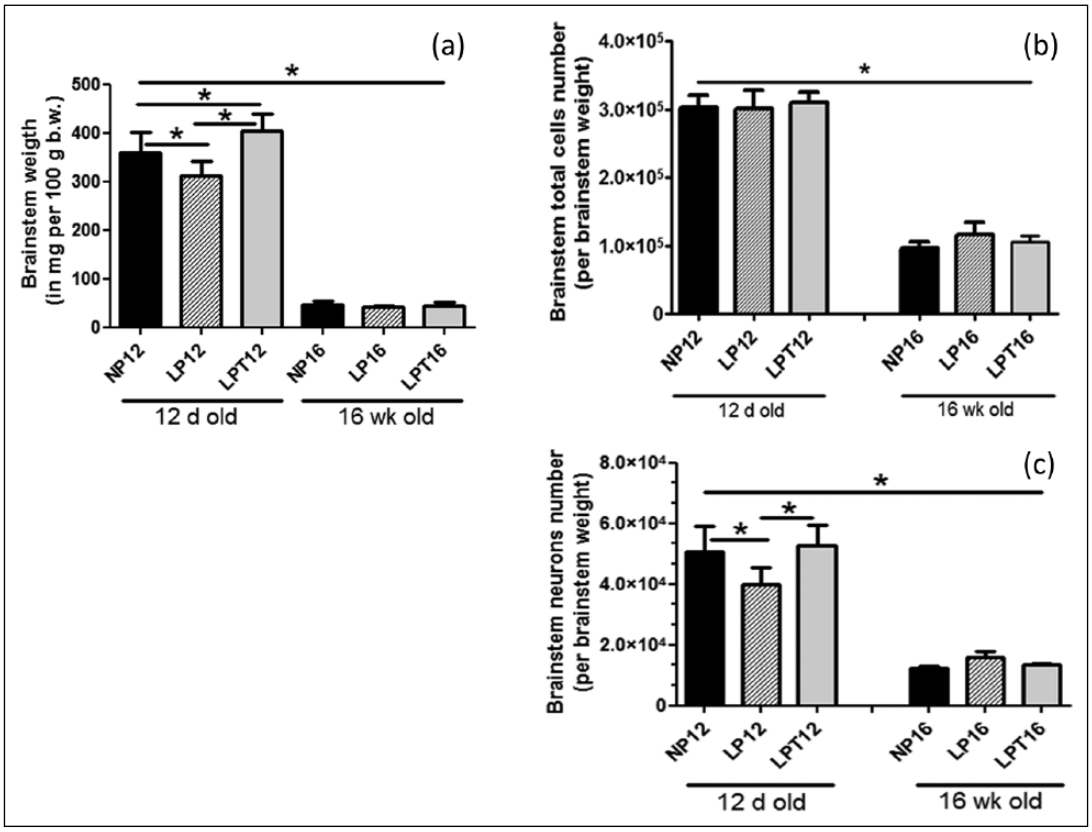

Figure 2. Effect of age on brainstem weight (per 100 g body weight, 3(a), on top); brainstem total cellular quantification (per mg of the brainstem mass, 3(b), on top) and, brainstem neurons quantification (per mg of the brainstem mass, 3(c), on bottom) in male offspring of protein-restricted, taurine-supplemented and standard-diet mothers during gestation. See Results for statistical analysis details. The data are reported as the means ± SD. In all experiments, n = 6; *p ≤ 0.05 vs. NP (analysis of variance (ANOVA); post hoc Bonferroni's test).

when compared to LP weight gain (LP1 vs. LP3: 32.75%, $p < 0.01$). The data relating to offspring body weights were obtained on a daily basis. However, in this study data were presented as the weekly average of all daily weights. In general, food intake and therefore sodium intake were similar, when normalized by body weight, in male offspring of NP, LP, and LPT groups during the investigation. The LP male pup body weight was significantly reduced when compared to that of NP and LPT pups (5.98 ± 0.6 g vs. 6.65 ± 0.46 g and 5.98 ± 0.6 g vs. 6.58 ± 0.32 g, respectively, $p = 0.005$). However, the body masses at 16 weeks old ($p > 0.05$) were similar to those observed in NP and LPT age-matched groups (Table 1). The arterial blood pressure increased significantly more in LP than in NP rats from 6 to 16 weeks of age; LP pressure increased from 116.2 ± 6.5 mmHg to 137.9 ± 6.9 mmHg as compared with a smaller and nonsignificant rise from 114.7 ± 7.4 mmHg to 118.8 ± 8.7 mmHg and from 114.0 ± 7.4 mmHg to 123.2 ± 5.6 mmHg (n = six for each group; $p = 0.01$ vs. NP) in NP and LPT, respectively. In LP, the significant rise in the arterial pressure appeared after 12 weeks of age (Figure 2).

Total cell and neuron quantification of the medulla oblongata

The LP male pup (12 days of age) brainstem weight, normalized by body weight for each experimental group, was

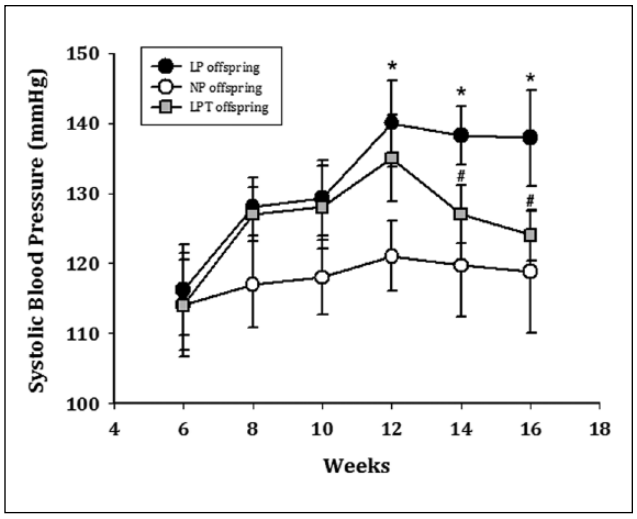

Figure 3. Effects of age on arterial pressure in male offspring of protein-restricted, taurine-supplemented and standard-diet mothers during gestation. See results for statistical analysis details. The data are reported as the means ± SD. In all experiments, n = 6; *$p \leq 0.05$ vs. NP (analysis of variance (ANOVA); post hoc Bonferroni's test).

significantly reduced when compared to that of NP (less about 15%) and LPT (less about 29%) pups (311 ± 31.7 mg% vs. 358.9 ± 41.9 mg% and 311 ± 31.7 mg% vs. 404 ± 34.1 mg%; $p = 0.03$), respectively. However, the brainstem masses at 16 weeks old ($p > 0.05$) per 100 g of body weight were similar to those observed in NP and LPT age-matched offspring (Figure 3(a), on top). We find a similar total number of cells per brainstem weight (in mg) in 12-day and 16-week of age-matched NP adult offspring (12-day: $33.5 \pm 7.3 \times 10^4$; 16-week: $13.5 \pm 2.5 \times 10^4$ cells; n =five) when compared to medulla oblongata of the LP (12-day: $27.4 \pm 73.5 \times 10^4$; 16-week: $11.73 \pm 1.4 \times 10^4$ cells; $n = 5$) and LPT (12-day: $31.7 \pm 5.1 \times 10^4$; 16-week: $9.48 \pm 3.21 \times 10^4$ cells; n = five) (Figure 3(b), on top). However, the brainstem total cells for 12-day- and 16-week-old ($p > 0.05$) per mg de medulla oblongata were similar to those observed in NP and LPT age-matched offspring, there is a significant difference between 12-day and 16-week-old groups (Figure 3(a), on top). Therefore, the pup (12-day-old) rat contains almost three times as many cells per mg of the brainstem (averaging $30.42 \pm 2.01 \times 10^4$ cells) as the brainstem of 16-week-old offspring (averaging $10.62 \pm 1.2 \times 10^4$ cells, $p < 0.05$). Conversely, the neuron quantification per brainstem weight (in mg) shows an expressive reduction of neurons (about 21%) in 12-day-old offspring ($40 \pm 5.55 \times 10^3$ neurons, $p < 0.05$) when compared to NP ($50.8 \pm 8.43 \times 10^3$ neurons) and LPT ($52.5 \pm 7.03 \times 10^3$ neurons) offspring (Figure 3(c), on bottom). Otherwise, no difference was observed among LP, NP and LPT groups at 16-week-old rates. Therefore, the pup (12-day-old) offspring contain almost two and half times as many cells per mg of the brainstem (averaging $47.7 \pm 7.0 \times 10^3$ neurons) than in the brainstem of 16-week-old offspring (averaging $13.9 \pm 1.04 \times 10^3$ neurons, $p < 0.05$).

Renal function data

The data for renal function in the 8- and 16-week-old offspring of the NP, LP, and LPT groups are summarized in Figure 3. The urinary flow rates (Table 1) and the GFR, estimated by C_{Cr}, after oral water load, did not significantly differ among the groups during the renal tubule sodium handling studies. FE_{Na} was significantly lower in both LP ($0.74 \pm 0.03\%$) and LPT ($0.79 \pm 0.02\%$) offspring beyond 8 weeks old, when compared with the NP age-matched group ($0.95 \pm 0.07\%$; $p = 0.0001$). However, the FE_{Na} of 16-week-old offspring remained significantly decreased in the LP, but not in the LPT ($0.98 \pm 0.12\%$) group, as follows: LP 16-week: $0.72 \pm 0.03\%$ vs. NP 16-week: $1.05 \pm 0.1\%$ ($p = 0.0001$), respectively. The decreased FE_{Na} in LP offspring was accompanied by a significant decrease in FEP_{Na} when compared only with the NP age-paired control group ($p = 0.01$). This decreased FE_{Na} occurred despite unchanged C_{Cr} and a significant enhance in $FEPP_{Na}$ in the 8-week-old groups (LP: $1.08 \pm 0.13\%$ vs. NP: $0.84 \pm 0.11\%$, $p = 0.006$ and vs. LPT: $0.84 \pm 0.11\%$, NS), as well as in 16-week-old offspring (LP: $1.12 \pm 0.15\%$ vs. NP: $0.73 \pm 0.14\%$, $p = 0.002$ and vs. LPT: $0.92 \pm 0.16\%$, NS) (Figure 3). This consistent fall in FE_{Na} and FEP_{Na}, and the increase in $FEPP_{Na}$, produced by protein-intake restriction during pregnancy, was followed by unaffected kaliuresis in the entire experimental groups of the present investigation.

Western blot analysis of $AT1_R$ and $AT2_R$ expression

Western blot analysis in male offspring of NP, LP, and LPT rat brainstems in 12-day- and 16-week-old offspring yielded a single band at the expected weight of corresponding proteins. The expressions of AT_1R proteins studied in the whole medulla oblongata tissue extracts of 12-day- (NP: 100 ± 6.42%; LP: 52 ± 27.56% and LPT: 61.14 ± 19.1%, $p > 0.06$) and 16-week-old LP and LPT rats (Figure 4), despite being lower were not statistically significant when compared to those of NP and LPT rats (Figure 4). Additionally, the expression of the AT_2R proteins in the brainstem of 12-day- (NP: 100 ± 36.41%; LP: 81.35 ± 24.05% and LPT: 62.6 ± 14.5%) and 16-week-old LP and LPT offspring ($p = 0.489$) was unchanged when compared with the age-paired NP group (Figure 4).

nTS immunohistochemical analysis of AT_1R and AT_2R

The nTS AT_1R immunoreactivity was markedly decreased in 16-week-old LPs comparatively to the NP age-matched offspring group. Otherwise, in 16-week-old LPTs, the immunoreactivity to AT_1R presented similarly to that observed in NP offspring of the same age and in this similar brainstem nucleus (Figure 5). The unchanged AT_1R

blotting in the whole medulla oblongata extracts of 16-week-old LP rats may result in uneven AT_1R and AT_2R expression in several nuclei, as revealed by qualitative immunohistochemistry of different parts of analyzed nTS structures. At the same time, the nTS AT_2R immunoreactivity was markedly decreased in 16-week-old LP and LPT rats when compared with age-matched NP offspring (Figure 6).

Discussion

IUGR is a pregnancy complication associated with adverse outcomes such as neurodevelopmental handicaps.[20,21] The morpho-functional organization of the CNS in mammals is established during the prenatal and early postnatal periods of development through the synthesis of cellular components, neurogenesis, and gliogenesis, migration, and cell differentiation. Although the precise mechanism by which blood pressure rises in LP offspring remains to be elucidated, neural activity and renal control of the fluid and electrolyte balance are thought to play a dominant role in the long-term control of arterial blood pressure. In uteri programming of arterial hypertension via alteration of the RAS before birth has attracted great attention. The present study shows that the maternal body weight gain during the gestational period was significantly higher in NP and LPT when compared to LP weight gain. These maternal findings are associated with a significantly reduced LP male pup body weight compared to that of NP and LPT pups. Our data, confirming a prior report, indicate that LP kidneys even after higher blood pressure development excrete lesser amounts of salt under basal conditions than kidneys of NP rats.[19,22] Also, the present findings confirm previous studies in different areas of CNS,[23] but not in the brainstem, showing that maternal LP restriction during prenatal life decreases the mass and neuronal proliferation (about 21%) in this encephalic area. These disorders of the fetal brain areas, including the brainstem, may affect fetal neural cell maturation and hence have profound consequences in functional neural postnatal life. The nTS, the central site of termination of baroreceptive afferents, is intimately involved in arterial pressure control. This nucleus contains a high density of AT_1Rs located both presynaptically, on vagal and carotid sinus afferents, and on interneurons.[24] AngII lowers blood pressure and heart rate after injection of low doses in the nTS, as previously reported in several strains of rats.[9,10] This response was completely blocked by the nTS injection of an AT_1R antagonist.[11] The present study has shown a striking reduction of AT_1 receptors in the nTS of the maternal LP offspring when compared with unchanged AT_1R density in NP and taurine-treated LPT rats. The pattern of distribution of AT_1R has been identified on local neurons of the nodose ganglion and nTS as well as fiber terminals that project to these sites by anatomical, molecular or electrophysiological[24–26] techniques. Study has revealed that AngII

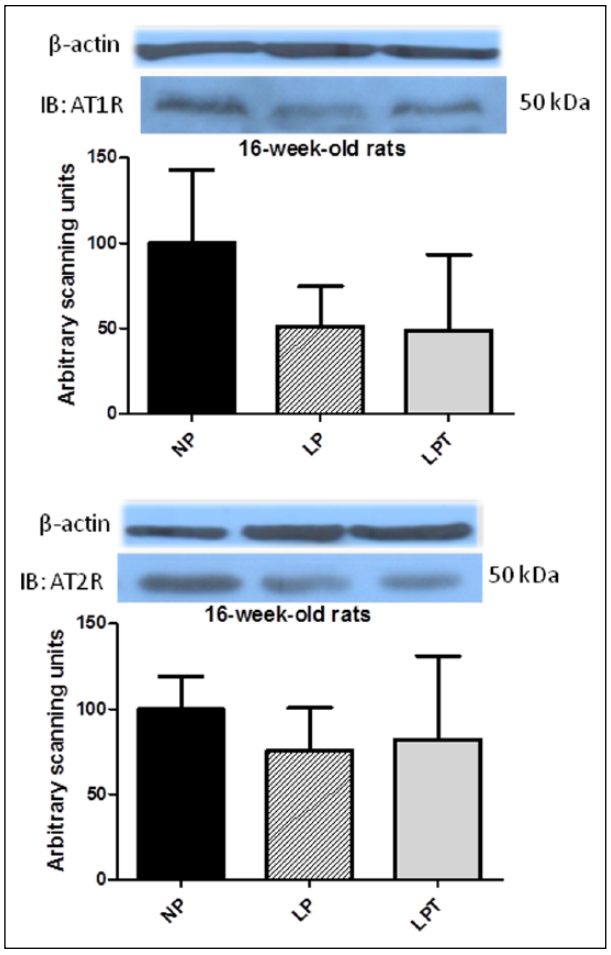

Figure 4. Effects of maternal protein restriction (LP) on expression of entire brainstem AT_1R and AT_2R proteins. This figure shows the results obtained in whole-tissue extracts that were immunoblotted for AT_1 and AT_2 receptors protein content verification in 16-week-old NP, LP, and LPT brainstems. The results of scanning densitometry were expressed as relative to NP, assigning a value of 100% to the control rats. AT_1R: type 1 AngII receptors; AT_2R: type 2 AngII receptors. Columns and bars represent the mean ± SD $*p < 0.05$ *vs.* NP (analysis of variance (ANOVA); post hoc Bonferroni's test).

may act directly on nTS neurons to stimulate efferent pathways responsible for parasympathetic control of blood pressure. However, at least in part, the AngII-mediated hypotensive effect may result from inhibition of sympathetic nervous system activity by direct connections of vagal sensory afferent fibers with cells in the A_2-catecholamine cell group in the ventral nTS.[27] We did not completely rule out that the blood pressure fall in LPT may have resulted from receptors on presynaptic afferent-fiber ends in the nTS that are most likely of vagal origin. In this case, studies have shown that substance P (SP) antagonists[10] attenuate the decreased pressure response elicited by AngII in the nTS.[28,29,30] The current study demonstrates a decreased nTS AT_1R expression, by immunohistochemistry, in 16-week-old

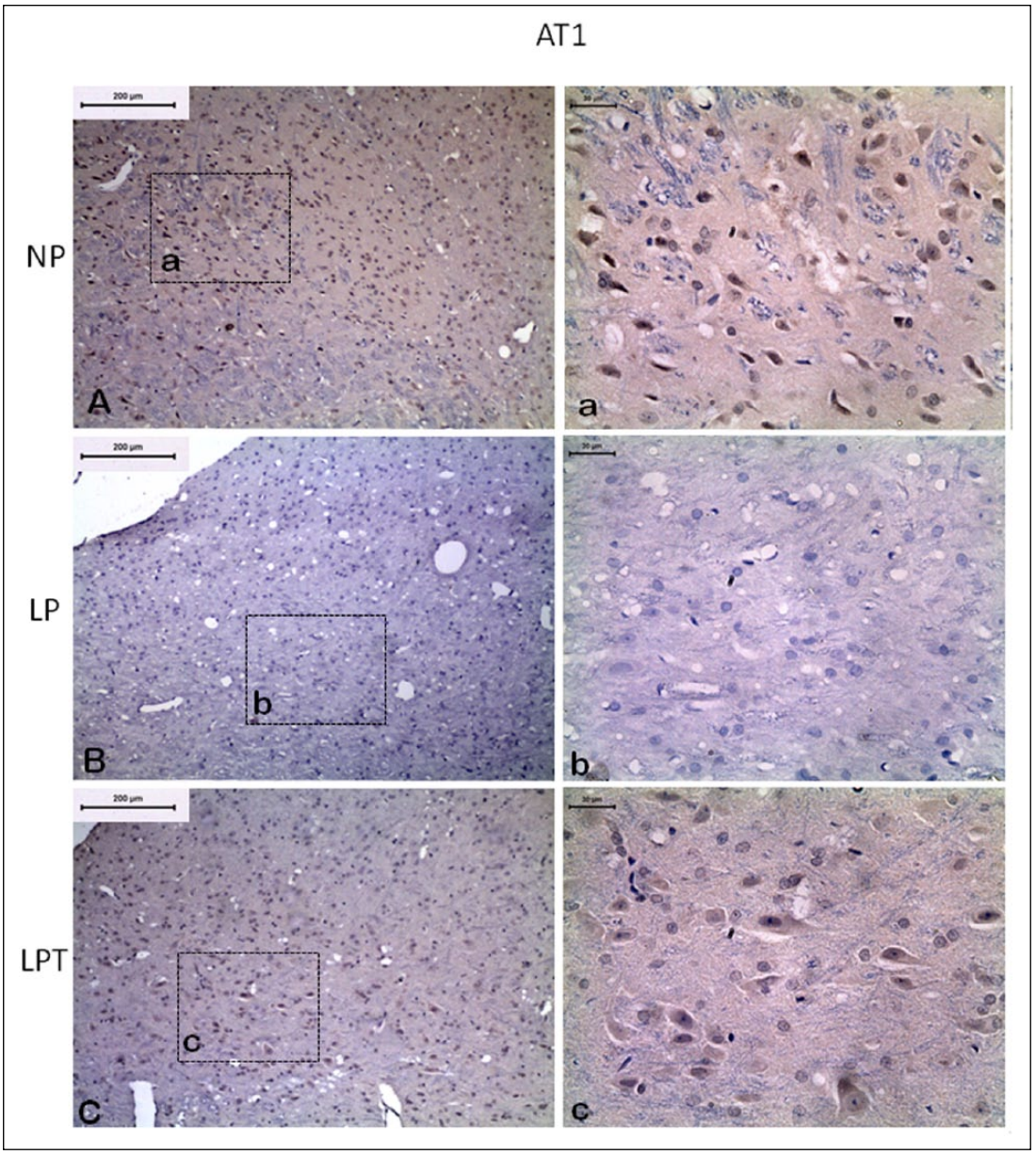

Figure 5. Effects of maternal protein restriction (LP) on AT_1R immunolocalization in 16-week-old rat transversal sections of tract solitary nucleus (nTS) compared to NP and LPT offspring. The immunoreactivity for this receptor was reduced in LP (B and b, small letter in detail), LPT (C and c) when compared to NP (A and a).[51] AT_1R: type 1 angiotensin II receptors; NP: normal protein; LPT: maternal protein restriction with taurine supplementation.

LP offspring when compared to NP and LPT groups. The nonsignificant decreased AT_1R blotting results, in the whole brainstem extracts of 16-week-old LP rats, may result in uneven angiotensin receptor expression in several subnuclei, as revealed by qualitative immunohistochemistry of rostral, medial and caudal subnuclei analyzed in nTS structures.

Maternal dietary protein restriction during pregnancy is associated with renal morphological and physiological changes. Different mechanisms can contribute to this phenotype: exposure to fetal glucocorticoid, alterations in the components of the RAS, apoptosis, and DNA methylation. An LP diet during gestation decreases the activity of placental 11ß-hydroxysteroid dehydrogenase, exposing the fetus to glucocorticoids and resetting the hypothalamic-pituitary-adrenal axis in the offspring. The abnormal function/expression of AngII receptors during any period of life may be the consequence or cause of renal adaptation. AT_1R is up-regulated, compared with controls, on the first day after birth of offspring born to LP diet mothers, but this protein appears to be down-regulated by 12 days of age and thereafter. In these offspring, AT_2R expression differs

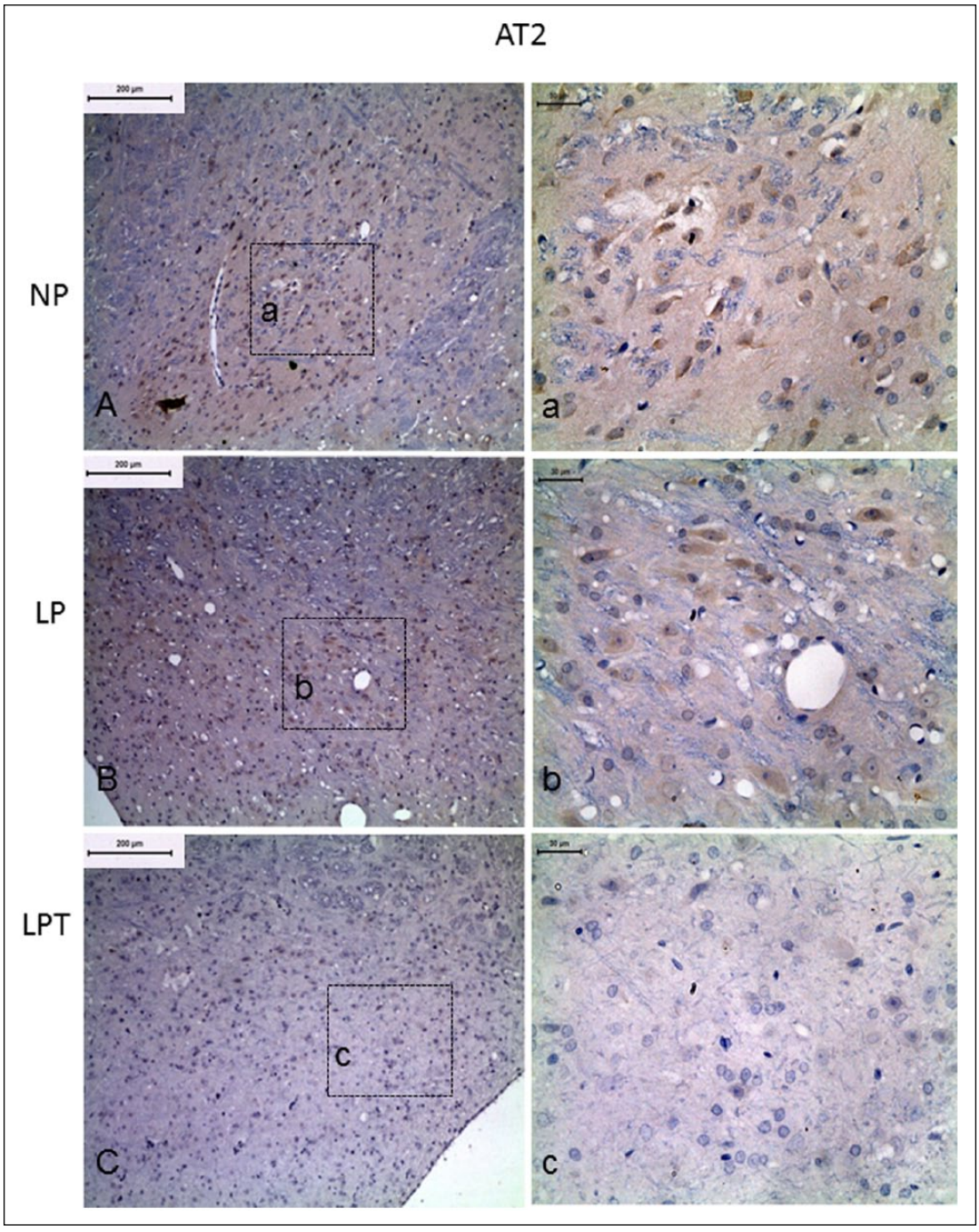

Figure 6. Effects of maternal protein restriction (LP) on AT_2R immunolocalization in 16-week-old rat transversal sections of tract solitary nucleus (nTS) compared to NP and LPT offspring. The immunoreactivity for this receptor was reduced in LP (B and b, small letter in detail) and LPT (C and c) when compared to NP (A and a).[51] AT_2R: type 2 angiotensin II receptors; NP: normal protein; LPT: maternal protein restriction with taurine supplementation.

from controls at one day of age, but is also down-regulated thereafter, with low nephron numbers at all ages: from the fetal period, at the end of nephron formation, and during adulthood.[19,20] The current investigation also shows an early and pronounced decrease in FE_{Na} in maternal LP offspring beyond 8 weeks of age when compared to age-matched NP. The decreased FE_{Na} was accompanied by a fall in FEP_{Na} and occurred despite unchanged C_{Cr} and an enhanced $FEPP_{Na}$. In this case, fluid is reabsorbed to the same degree, resulting in the concentration in the end of the proximal tubule being the same as in the beginning. In other words, the reabsorption in the proximal tubule is

isosmotic without a change in the plasma osmolality. These effects were associated with a significant extracellular isotonic expansion and supposedly enhance arterial blood pressure in the LP group, but the precise mechanism of these phenomena remains unknown. While circulating AngII tends to retain sodium by a direct renal action,[7] as well as through aldosterone release from the adrenal gland, stimulation of brain AngII receptors has been reported to induce natriuresis.[31,32] The mechanism by which central AngII induces its natriuretic effects remains to be elucidated. Several possibilities may be considered. First, the CNS may directly influence renal sodium excretion through neural routes. Secondly, hemodynamic factors may be responsible for the alterations in electrolyte excretion. Thirdly, natriuresis may result from fluctuations in the level of neural factors that influence tubular sodium handling. There is considerable evidence to support a role for the sympathetic nervous system in the control of urinary sodium excretion.[4,5] Otherwise, some neurons in the nTS that express AT_1R have polysynaptic connections to peripheral organs such as the kidney via renal sympathetic nerves.[33,34] A previous study reported that, in conscious rats, central AngII induces an immediate reduction in the efferent renal nerve and enhanced renin-angiotensin dipsogenic and natriuretic response.[17,35,36] Otherwise, further studies are needed to evaluate the repercussion of taurine supplementation directly on kidney morphology and development, and vascular reactivity.

The second major isoform of the angiotensin receptor, AT_2R, is widely expressed at high levels in fetal tissues, and decreases rapidly after birth.[37,38] Alterations in AT_2R signaling may change the delicate balance between growth stimulation and inhibition, leading to alterations in development. However, according to current and previous studies, there is no strong evidence for AT_2Rs in the medial nTS of the rat that would account for the residual actions of AngII. Also, it is plausible to consider that the decreasing AT_1R/AT_2R ratio associated with attenuated AngII hypotensive and natriuretic responses, mediated by neural pathways with origin in nTS, is implicated with the higher blood pressure levels in adult LP offspring. These findings may also occur by opposite action of AT_2Rs to AT_1Rs in specific brain areas.[39]

A study has also shown previously that the level of taurine is markedly reduced in the plasma of fetuses of dams fed an LP diet.[12] Additionally, taurine supplementation of the maternal LP intake restored to normal the fetal plasma taurine concentration.[13] This β-amino acid can be regarded as an essential amino acid during fetal life, because the capacity to synthesize taurine is low or absent in the human fetus.[40,41] Consequently, the fetus is dependent on highly efficient active placental transport for a continuous supply of this amino acid. Apart from this, the physiologic function of this amino acid remains elusive. In animal experiments, including primate models, taurine deficiency during pregnancy and lactation is associated with growth failure, abnormal cerebellar development, neurologic deficits, retinal degeneration, and cardiac damage.[42] The role of taurine protecting against oxidative damage has been described in a variety of cell types. This amino acid, possibly through its antioxidant activity and regulation of intracellular calcium flux, can prevent the death of endothelial cells.[43] Taking into account the above findings, we may suppose that progressive enhanced blood pressure beyond 8 weeks of age in LP offspring may be associated with that pronounced reduction in cellularity and AT_1R density in the nTS. Conversely, this finding reverted by diet taurine-supplementation (in LPT), normalizing the arterial pressure and urinary sodium excretion in adult offspring. Moreover, AngII has been also shown to stimulate Ca^{2+} cellular transport.[44,45]

Taurine may also reduce blood pressure through attenuation of peripheral AngII activity, enhancement of the kinin-kallikrein system in the kidney,[46,47] or decreasing levels of epinephrine and norepinephrine. Additionally, as observed in the current study, taurine supplementation effectively normalizes high blood pressure in the most common animal models of hypertension, including: spontaneously hypertensive rats (SHR),[48] deoxycorticosterone acetate (DOCA)-salt rats,[49] and Dahl-S rats.[50]

Conclusion

In conclusion, although the precise mechanism responsible for the subsequently enhanced sodium retention response in LP offspring rats is still unclear, the current data suggest that maternal low taurine ingestion may lead to changes in nTS cardiovascular and sympathetic nerve activity that are conducive to excess hydroelectrolytic tubule reabsorption, and that this might potentiate the programming of adult hypertension. This raises the possibility that taurine during the gestational period may inhibit several other actions of AngII through the regulation of an early step in the signaling pathway of AngII. In fact, in the present study it is plausible to suppose an association of decreasing nTS neuronal cellularity, AT_1R/AT_2R ratio, and water-electrolyte renal excretion with the higher blood pressure levels found in LP (taurine-deficient progeny), compared with age-matched NP and LPT offspring. The mechanisms by which the fetal programming causes these disorders remain unknown and further studies are needed in this regard.

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Conflict of interest

None declared.

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