EXPERIMENTAL STUDY

Effects of dehydration on endocrine regulation of the electrolyte and fluid balance and atrial natriuretic peptide-binding sites in perinatally malnourished adult male rats

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Abstract

Objective: The first aim of this work was to investigate, under basal conditions in adult male rats, the long-term consequences of perinatal maternal food restriction on the plasma concentrations of vasopressin (VP), aldosterone and atrial natriuretic peptide (ANP) and on plasma renin activity (PRA). Furthermore, under these same conditions, the hypothalamic VP gene expression as well as the density (Bmax), affinity (Kd) and gene expression of ANP receptors were determined in kidneys and adrenals. The second aim of this work was to examine the responsiveness to dehydration in perinatally malnourished rats. Thus, the latter parameters were studied in these rats after 72 h water deprivation.

Methods: This study was conducted on 4-month-old male rats from mothers exposed to 50% food restriction (FR50) during the last week of gestation and lactation and on age-matched control animals (C). At this stage, both C and FR50 rats were killed by decapitation between 0900 h and 1000 h in order to determine parameters under basal conditions or after 72 h water deprivation. Plasma VP, ANP and aldosterone levels and PRA were determined by radioimmunoassay. Hypothalamic VP gene expression was determined in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) by in situ hybridization. The Bmax and Kd values of ANP receptors were evaluated from Scatchard plots. ANP receptor gene expression was determined by Northern blot analysis.

Results: Under basal conditions, perinatal malnutrition reduced body weight, absolute weight of kidneys and adrenals, and haematocrit. Compared with control rats, FR50 rats had significantly greater plasma VP and aldosterone levels but PRA, plasma ANP levels, plasma osmolality and hypothalamic VP gene expression were not significantly different. Perinatal malnutrition did not significantly affect glomerular ANP receptor density, but in adrenals it decreased both Bmax and Kd values of ANP-B receptors (biological receptors) and increased Bmax of ANP-C receptors (clearance receptors). ANP-Bα (receptor subtype A of ANP-B receptors) receptor gene expression was not significantly affected, whereas ANP-C receptor gene expression was enhanced in both adrenals and kidneys in FR50 rats. After 72 h dehydration, control rats showed a significant rise in haematocrit, plasma osmolality, PRA, circulating levels of VP and aldosterone and VP gene expression in both PVN and SON but showed a decrease in plasma ANP levels. Bmax of ANP-B receptors was decreased whereas Bmax of ANP-C receptors was enhanced in both adrenals and kidneys. ANP-Bα receptor gene expression was not significantly affected in either kidneys or adrenals in dehydrated control rats. Similarly, ANP-C receptor gene expression was unaffected in kidneys whereas it was significantly enhanced in adrenals. In FR50 rats, the effects of water deprivation were qualitatively similar to those reported in controls concerning plasma parameters except for plasma VP levels which tended to rise (not significant) but this increase was only very slight compared with controls. Moreover, unlike controls, VP gene expression in both PVN and SON was not enhanced after dehydration in FR50 rats. In kidneys, dehydrated FR50 rats, like controls, upregulated ANP-C receptors, but they were unable to downregulate ANP-B receptors. In adrenals, unlike controls, FR50 rats enhanced ANP-B receptor density whereas they decreased both ANP-C receptor density and expression after 72 h dehydration. Similar to controls, the expression of ANP-Bα receptors in both kidneys and adrenals as well as the expression of ANP-C receptors in kidneys, were unaffected in dehydrated FR50 rats.

Conclusion: Perinatal malnutrition had long-lasting effects on regulation of the fluid and electrolyte balance under basal conditions. The main effects were a significant rise in circulating levels of VP and aldosterone, and changes in density of adrenal ANP-binding sites and ANP-C receptor gene expression in both adrenals and kidneys. Perinatal malnutrition also affects the responsiveness to water deprivation with alterations in both hypothalamic VP gene expression and regulation of ANP-binding sites.

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Introduction

Regulation of body fluid homeostasis within narrow limits depends on maintenance of water and electrolyte (particularly sodium) balance. Maintenance of body fluid balance under a wide range of conditions is controlled primarily by vasopressin (VP) which acts mainly as an antidiuretic hormone (1). Sodium balance is controlled primarily by the renin–angiotensin system (2) and aldosterone (3, 4). Atrial natriuretic peptide (ANP), a 28 amino acid peptide synthesized by mammalian atrial cardiomyocytes, is also involved in the regulation of body fluid and electrolyte balance (5). ANP is the main hormone released in response to blood volume expansion. It counteracts the actions at the target organ of both the renin–angiotensin–aldosterone system and VP. Indeed, ANP stimulates natriuresis and diuresis by enhancing glomerular filtration rate and excretion of sodium and water (6) and inhibiting the secretion of aldosterone induced by angiotensin II (7), renin (8) and VP (9). Subsequently, two other natriuretic peptides with a significant sequence homology with ANP have been identified and named brain natriuretic peptide (BNP) (10) and C-type natriuretic peptide (CNP) (11). These three natriuretic peptides express their functions by interaction with specific binding sites, which have been classified into biologically active receptors (ANP-B receptors) and clearance receptors (ANP-C receptors). ANP-B receptors are coupled to guanylate cyclase (GC) and mediate the biological effects of natriuretic peptides. Two subtypes of GC natriuretic receptors have been cloned and named A and B receptors (12, 13). The A receptor (ANP-B<sub>A</sub> receptor) has a higher affinity for ANP, an equal or lower affinity for BNP and a significantly lower affinity for CNP. The B receptor (ANP-B<sub>B</sub> receptor) has a higher affinity for CNP and a lower affinity for BNP and ANP (14, 15). ANP-C receptors have equal affinity for ANP, BNP and CNP and lack an intracellular guanylate cyclase domain, and function as clearance receptors for all three natriuretic peptides (16).

Intrauterine growth retardation (IUGR) is a common complication of pregnancy and a significant cause of perinatal morbidity and mortality. Barker et al. (17) reported that fetal malnutrition causes persistent endocrine changes leading to abnormal structure, function, and disease in adult life. Indeed, epidemiological evidence suggests that a low birth weight increases the risk of developing a number of adult-onset disorders, including non-insulin-dependent diabetes mellitus, hypertension, and cardiovascular diseases (17, 18). Regulation of body fluid homeostasis may also be affected by perinatal malnutrition as suggested by several studies of fetal growth restriction in humans or animals. In humans, renin gene expression persists and predominates in the deep renal cortex of the stillborn IUGR fetus. The normal shift in renin gene expression to the superficial cortex of the kidney does not occur. These observations may represent altered programming of fetal renin gene expression in the IUGR fetus (19). In sheep, restriction of nutrient and oxygen supply during fetal life was associated with suppression of renal renin and renal angiotensinogen gene expression in fetuses (20) and enhanced plasma renin activity in one-week-old lambs (21). In rats, perinatal protein restriction suppresses the newborn intrarenal renin–angiotensin system (22). At weaning, plasma renin activity was elevated whereas plasma angiotensin II concentrations were decreased in rats exposed to maternal low-protein diets (23). In other studies, rat offspring of low-protein diet pregnancies exhibited enhanced plasma aldosterone levels at four and eight weeks of age (24) or increased haematocrit at 21 weeks of age (22).

IUGR has also been linked to impaired renal growth, development and function. Indeed, during the first week of life, diuresis was lower whereas urinary sodium excretion was slightly higher in very low birthweight infants (25). Furthermore, in infants, IUGR is also associated with a reduction in the number of glomeruli (26, 27). In adult progeny of rats submitted to 50% dietary restriction during pregnancy, a significant decline in glomerular filtration rate, renal plasma flow and in the number of glomeruli occurred (28). Moreover, in these rats, glomerular diameter was increased (29), the percentage of glomeruli with sclerosis was greater and there was more intense tubulointerstitial lesions and immunohistochemical alterations in the renal cortex (30). Impairment of glomerulogenesis could lead to decreased filtration of sodium, increased extracellular volume and consequently development of hypertension (31–33). Taken together, these investigations show that the regulation of body fluid homeostasis in adulthood could be impaired by an exposure to perinatal undernutrition. So, the first goal of this study was to investigate the long-term consequences of perinatal maternal malnutrition on plasma concentrations of VP, ANP and aldosterone and on plasma renin activity, under basal conditions in adult male rats. Furthermore, under these same conditions, the hypothalamic VP gene expression as well as the density, affinity and gene expression of ANP receptors were determined in adrenals and kidneys.

Dehydration is a classic homeostatic challenge that leads to a series of well-characterized endocrine responses. These are directed towards normalizing the volume and osmolality of the extracellular fluid compartment as rapidly as possible. Thus, many studies in rats have shown that dehydration enhances plasma VP levels (34, 35) as well as hypothalamic VP gene expression (34, 36). Dehydration also causes a rise in plasma aldosterone levels (37), a fall in plasma ANP concentrations (38, 39) and an increase in ANP-C receptor density in kidneys (38). It is not known if perinatal maternal malnutrition alters these homeostatic responses to dehydration. Therefore, the second aim...
of this study was to examine, in rats exposed to perinatal maternal malnutrition, plasma concentrations of VP, ANP and aldosterone and plasma renin activity after 72 h dehydration. Hypothalamic VP gene expression as well as the density, affinity and gene expression of ANP receptors in adrenals and kidneys were also determined under these experimental conditions.

Materials and methods

Animals and housing conditions

Wistar rats (200 g) were purchased from IFFA-CREDO (L’Arbresle, France) and housed five per cage in a room with a controlled light cycle (12 h light, 12 h darkness, lights on at 0700 h) and temperature (22 ± 2 °C), with free access to food (regular rat chow no. 113, containing 22% protein, 5% fat, 53% carbohydrates; UAR, Villemoisson-sur-Orge, France) and tap water. After 8 days of acclimation, females were mated with a male for one night. The following day was taken as day 0 of pregnancy if spermatozoa were found in vaginal smears. Pregnant females were then transferred to individual cages. Animal use accreditation by the French Ministry of Agriculture (no. 04860) has been granted to our laboratory for experimentation with rats.

Feeding regimens and study design

Each pregnant dam was randomly assigned to the control (n = 20) or the food-restricted group (n = 20). Control pregnant dams had food available ad libitum, whereas pregnant dams from the food-restricted group (FR50) were fed daily with 12 g commercial rat chow, which represents about 50% of the daily intake of control pregnant dams, from day 14 of pregnancy onwards. Maternal food restriction was continued until the end of lactation as previously described (40). At parturition, litter size was adjusted to 8 pups per dam in both control and food-restricted mothers. After weaning, all offspring were caged individually and food was available ad libitum until adulthood. Adult male offspring from the control (C) or the food-restricted (FR50) group were studied at 4 months of age. At this stage, animals were randomly divided into two groups. In the first group, both C (n = 24) and FR50 (n = 24) rats were killed by decapitation between 0900 h and 1000 h in order to determine parameters under basal conditions. In this group, tap water was available ad libitum. In the second group, both C (n = 24) and FR50 (n = 24) rats were killed by decapitation after 72 h water deprivation. Only a limited number of offspring (n = 1 - 3) was used from each litter to obviate a putative litter effect.

Plasma and tissue collections

Trunk blood samples of rats were collected after decapitation in prechilled polyethylene tubes containing 20 μl 5% EDTA for 1 ml blood. A blood sample (0.5 ml) was taken for haematocrit determination and the remainder was centrifuged at 5000 g for 10 min at 4°C. Plasma samples were frozen at −80°C until measurement of osmolality, VP, ANP, aldosterone and renin activity.

Adrenals and kidneys were quickly removed, weighed and thereafter rapidly frozen in liquid nitrogen before being stored at −80°C until extraction of mRNA for Northern blot analysis or preparation of tissue samples for binding assay. Adrenals used for binding assay were first decapsulated in order to separate the capsule with the associated zona glomerulosa from the inner tissue. For in situ hybridization studies, brains were removed, immediately frozen on dry ice and stored at −80°C until sectioning.

Preparation of tissue samples for ANP receptor binding assay

For each experiment, six adrenals or four kidneys were pooled to prepare tissue samples for one binding assay. Capsules with associated zona glomerulosa were ground with a Teflon-glass homogeniser in Tris–HCl buffer (50 mmol/l, pH 7.4) containing MnCl₂ (5 mmol/l) and NaCl (120 mmol/l). The homogenates were centrifuged at 1000 g for 20 min at 4°C. The supernatants were collected and centrifuged at 20 000 g for 30 min at 4°C. The pellets containing the membranes were rinsed with Tris–HCl buffer and centrifuged again at 20 000 g for 10 min at 4°C. The supernatants were discarded and the pellets diluted with Tris–HCl buffer. An aliquot of the pellet suspension was taken for protein determination and the remainder frozen in liquid nitrogen and stored at −80°C until required for binding assay.

Glomeruli were prepared according to the technique previously described by Sraer et al. (41). Briefly, the kidneys stripped of their renal capsule were cut longitudinally in order to remove the inner tissue. The cortical tissue was carefully minced and passed successively through a 106 μm metal sieve, which excluded the tubules and blood vessels, and a 53 μm metal sieve, which retained the glomeruli. The glomerular suspensions were rinsed twice with ice-cold Tris–HCl buffer, centrifuged at 1000 g for 5 min at 4°C and then diluted with Tris–HCl buffer. The purity of the preparation was checked by light microscopy and tubular fragments were always less than 2% of the total number of glomeruli. An aliquot of the suspension was taken for protein determination and the remainder frozen in liquid nitrogen and stored at −80°C until required for binding. Protein content was determined by the method of Lowry et al. (42) using bovine serum albumin (BSA) as standard.
ANP receptor binding assay

Binding assay was performed according to the method developed in the laboratory and previously described (43, 44). Briefly, aliquots (50 μl) of adrenal membrane suspensions (1 mg/ml protein) or glomerular suspensions (2 mg/ml protein) were incubated in a final volume of 250 μl Tris–HCl buffer (50 mmol/l, pH 7.4) containing 5 mmol/l MnCl₂, 120 mmol/l NaCl, 1 μmol/l aprotinin, 0.5% BSA, 25 μmol/l 125I-rANP₁–28 (Amersham, UK) and increasing concentrations of unlabelled rat atrial natriuretic peptide (rANP₁–28) (Peninsula Laboratories, Belmont, CA, USA) ranging from 10⁻¹¹ to 10⁻⁴ mol/l. For the determination of the total population of ANP receptors. The density of ANP-B receptors was assessed with rANP₁–28 in the presence of an excess of cANP₁–23 (0.1 μmol/l; Peninsula Laboratories), a truncated ANP peptide which binds selectively to ANP-C receptors. The density of ANP-C receptors was obtained by subtracting the number of ANP-B receptors from the total ANP receptor population. Non-specific binding was measured in the presence of an excess of rANP₁–28 (0.1 μmol/l) and the specific binding was obtained by subtracting non-specific binding from the total binding. Binding studies were performed at room temperature. At the end of 60-min incubation period, the separation of free and bound 125I-rANP₁–28 was achieved by rapid filtration through (Whatman) GF/C filters (Prolabo, Marcq-en-Barœul, France), pre-soaked for at least 1 h in 0.2% polyethyleneimine. The filters were washed with 0.9% NaCl and counted in an LKB gamma counter.

The density (Bₜₐₜ) and affinity (Kₛ) of ANP receptors were evaluated from Scatchard plots (45).

Northern blot analysis for ANP receptors

Probes for hybridization Total RNA was extracted from 100 mg whole adrenal or kidney using Tri-Reagent (Sigma), which was a mixture of guanidine thiocyanate and phenol in a mono-phase solution. This procedure is an improvement of the single-step method reported by Chomczynski & Sacchi (46) for total RNA isolation. The purity and the yield of RNA were determined by the ratio of absorbance at 260 nm and 280 nm. Polyadenylated mRNA was purified via two cycles of oligo dT (deoxythymidine)-cellulose column chromatography (Miniprep Indirect Quick Messenger RNA, Talent, Trieste Italy).

As ANP-B₄ receptors have higher affinity for ANP than ANP-B₂ receptors, we studied herein only the expression of ANP-B₄ receptors and ANP-C receptors. The cDNA probes for ANP-B₄ and ANP-C receptors were generated by reverse transcriptase (RT) followed by the DNA polymerase chain reaction (PCR) using rat adrenal or kidney mRNA as the template (Access RT-PCR system, Promega, USA). The sequences of synthetic primers (Eurogentec, Seraing, Belgium) used for PCR amplification were: for ANP-B₄ receptors 5’-AAG-AGCCTGAATACTCTGATCT-3’ (forward) and 5’-TTGCAGGAAGTGTCCTCATTGTCA-3’ (reverse: from rat sequence (47)), and the predicted length of the PCR product was 451 bp. For ANP-C receptors, the sequences of primers were 5’-ATGTGGGCGCATCCAGGCGAGT-3’ (forward) and 5’-TCAAAAGTATAATCACCATAAActCTGCTGGTACCACG-3’ (reverse: from bovine sequence (48)) and the predicted length of the PCR product was 573 bp. For PCR amplification, the thermal cycle program consisted of an initial incubation at 94 °C for 2 min followed by 40 cycles (94 °C for 30 s, 60 °C for 1 min and 68 °C for 2 min) and a final extension at 68 °C for 7 min. Amplified cDNA was size-fractionated by electrophoresis on agarose gels containing ethidium bromide, observed under UV light and extracted using QIA quick gel extraction kit (Qiagen, Hilden, Germany). The cDNA probe for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was a generous gift from Professor J J Curgy (Université de Lille 1, France).

Northern blot hybridization Aliquots of the total RNA extract (20 μg) from adrenals or kidneys were vacuum dried and denatured in 5 μl formamide. Samples of total RNA were then fractionated on 0.8% agarose gels containing 2.2 mol/l formaldehyde and MOPS buffer (20 mmol/l 3-(N-morpholino) propane sulphonic acid, pH 7.0, 5 mmol/l sodium acetate, 1 mmol/l EDTA). After electrophoresis, RNA was capillary transferred to hybrid-N nylon membranes (Amersham) in 20 ml SSC (saline sodium citrate, 1 x SSC = 0.15 mol/l NaCl and 0.015 mol/l citrate). The membranes were dried at room temperature for 1 h and baked at 80 °C for 2 h, in order to immobilize RNA. Membranes were then hybridized sequentially with ANP-B₄ or ANP-C receptor probes and GAPDH probe. The GAPDH mRNA level in each sample was used as an internal standard because it was not affected under our experimental conditions. Hybridizations were performed with cDNA probes labelled with [α³²P]dCTP (3000 Ci/ml, Amersham) by the random prime labelling system (Rediprime II, Amersham) and purified on ProbeQuant G-50 Micro Columns (Amersham). Blots were prehybridized for 4 h at 42 °C in 50% formamide, 5 × PBS-EDTA (SSPE), 5 × Denhardt’s, 1% sodium dodecyl sulphate (SDS) and denatured salmon sperm DNA (200 μg/ml), then hybridized in the same buffer except for the ANP-B₄ receptor probe which was hybridized in the presence of 10% dextran-sulphate. Hybridization with ³²P-labelled ANP-B₄ receptor probe (1.5 x 10⁶ c.p.m./ml) or ANP-C receptor probe (10⁶ c.p.m./ml) and GAPDH probe (10⁶ c.p.m./ml) was accomplished for 16 h at 42 °C. For ANP-B₄ receptor mRNA determination, blots were washed once in 2 x SSC/0.1% SDS for 5 min at room temperature and then twice in 0.2 x SSC/0.1% SDS for 5 min at 45 °C. For ANP-C receptor mRNA determination, blots were washed once in 0.2 x SSC/0.1% SDS for 15 min at 45 °C.
room temperature, followed by a 15 min wash at 45°C using the same solution and finally 30 min at 65°C with 0.1×SSC/0.1% SDS. For GAPDH mRNA determination, blots were washed once in 2×SSC/0.1% SDS for 20 min at room temperature, then twice in the same solution for 5 min at 55°C and finally twice in 0.1×SSC/0.1% SDS for 5 min at 55°C. The membranes were exposed to hyperfilm MP X-ray (Amersham) with intensifying screen at −80°C (1–3 days). Hybridization signals were quantified on the film autoradiograms. The optical density (OD) of the hybridized signal was measured using a GS-700 densitometer coupled with a computer-assisted image analysis using Multi-Analyst software (Bio-Rad Laboratories, Hercules, CA, USA). Blots were normalized with signals from GAPDH to correct for variation, efficiency of RNA transfer. So, values for ANP-Bα and ANP-C receptor mRNAs were expressed relative to GAPDH.

In situ hybridization for hypothalamic VP mRNA

Coronal sections (12 μm) of the brain through the hypothalamus were made with a cryostat at −20°C. The sections were mounted onto two times gelatin-coated slides, dried on a slide warmer and kept at −80°C.

In situ hybridizations were performed as previously described (49). The VP probe was a 200-bp EcoRI-BamHI fragment of the rat VP cDNA (kindly provided by Drs C Rabadan-Diehl and G Aguilera, NICHD, NIH, Bethesda, MD, USA) and linearized with BamHI. Riboprobes were labelled using [35S]UTP (1300Ci/mmol, NEN Life Science Products, Paris, France) and synthesized according to the previously detailed procedure (50).

Three sections of the brain from each animal were analysed. Hybridization signals were quantified on the film autoradiograms. The optical density (OD) of the hybridized signal was measured in both the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus and expressed as OD/mm².

Measurement of plasma parameters

Plasma VP concentrations were measured by radioimmunoassay (RIA) after extraction from 1 ml plasma sample through Sep-pak C18 cartridges and the adsorbed peptide was eluted with 3 ml 60% acetonitrile in 1% trifluoroacetic acid (TFA). The percentage recovery of a known amount of labelled ANP was 81%. ANP levels were measured with an RIA commercial kit (RIK 9103, Peninsula Laboratories, INC., San Carlos, CA, USA) using a specific ANP antiserum which had no cross-reactivity with BNP-32 (human), BNP-45 (rat) and CNP-22 (porcine). Plasma renin activity was measured as the rate of generation of angiotensin I (ANGI) in ng/ml plasma/h by renin acting on endogenous substrate, at pH 5.5–6.0 and at 37°C. Angiotensin I concentrations were determined by an RIA kit (REN-CT2, CIS bio international, Gif-Sur-Yvette, France), using a specific ANGI antiserum which had less than 0.01% cross-reactivity with angiotensin II and angiotensin III. The intra- and interassay coefficients of variation were 5.27% and 7.57% respectively. The aldosterone assay in plasma samples was preceded by extraction with ethylacetate after delipidation with isooctane. Recovery of labelled aldosterone from plasma was over 95%. The RIA method for aldosterone has been described previously (51). The specific aldosterone antiserum used had less than 0.001% cross-reactivity with deoxycorticosterone, progesterone and testosterone. The intra- and interassay coefficients of variation were 5.06% and 10.26% respectively.

Haematocrit was determined by the microcapillary technique (Clay-Adams microhaematocrit). Plasma osmolality was measured by depression of freezing-point (Roebling osmometer).

Statistical analysis

All data are presented as means±S.E.M. Statistical analysis was performed using analysis of variance (ANOVA) on two factors: perinatal maternal regimen (C or FR50) and condition (basal or dehydration). Dunnett’s test was used for post hoc comparisons. A P value <0.05 was considered statistically significant.

Results

Body and organ weights and blood parameters

Under basal conditions, 4-month-old FR50 rats exhibited reduced body weight (P < 0.001) and absolute weight of kidneys (P < 0.001) and adrenals (P < 0.01). In contrast, the relative adrenal weight was significantly (P < 0.05) increased in FR50 rats whereas the relative kidney weight was not affected (Table 1). The haematocrit was significantly decreased (P < 0.05) whereas plasma osmolality was not significantly affected (Table 1).

Water deprivation for 72 h induced in both control and FR50 rats significant (P < 0.001) decreases in body weight but increases in relative weight of kidneys (P < 0.001) and adrenals (P < 0.001 and P < 0.01
Table 1 Body weight (BW), absolute and relative weight of kidneys and adrenals, haematocrit and plasma osmolality under basal conditions and after 72 h dehydration in 4-month-old male rats from mothers fed ad libitum (control, C) or subjected to a 50% food restriction during the last week of gestation and during lactation (FR50). Values are means ± S.E.M. (n = 9 animals per group).

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Basal conditions</th>
<th>After 72 h dehydration</th>
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<tbody>
<tr>
<td></td>
<td>Body weight (g)</td>
<td>Kidneys (g)</td>
</tr>
<tr>
<td>C</td>
<td>472.47 ± 7.02</td>
<td>2.54 ± 0.05</td>
</tr>
<tr>
<td>FR50</td>
<td>395.93 ± 9.12</td>
<td>2.16 ± 0.04</td>
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<tr>
<td></td>
<td>409.56 ± 5.45</td>
<td>2.42 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>2.05 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>FR50</td>
<td>0.63 ± 0.01</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01 FR50 vs respective control; ***P < 0.001 after dehydration values vs respective basal values.

respectively), haematocrit (P < 0.05 and P < 0.01 respectively) and plasma osmolality (P < 0.001 and P < 0.01 respectively). Nevertheless, 72 h water deprivation was unable to markedly affect absolute weight of kidneys in either group and a significant perinatal regimen × condition interaction (F1,32 = 9.85, P = 0.004) was found on the absolute weight of adrenals which was enhanced (P < 0.01) only in controls (Table 1). Under these conditions, FR50 rats showed lower (P < 0.001) body weight and absolute weight of kidneys and adrenals but higher relative kidney weight (P < 0.001) and plasma osmolality (P < 0.05) than controls (Table 1).

**Plasma hormone levels**

Under basal conditions, rats exposed to maternal food restriction showed significant increases in plasma VP (P < 0.05) and aldosterone (P < 0.01) levels (Table 2). In contrast, baseline levels of plasma ANP and renin activity were not significantly affected by perinatal food restriction (Table 2).

A significant interaction between perinatal regimen and condition (F1,32 = 5.44, P = 0.026) was found on plasma VP levels. Thus, whereas dehydration for 72 h induced in controls a marked (P < 0.001) increase in plasma VP levels (31.7-fold), the dehydration-induced rise in plasma VP levels was slight (1.7-fold) and insignificant in FR50 rats (Table 2). Water deprivation caused a significant (P < 0.001) decrease in plasma ANP levels and a marked increase in plasma aldosterone levels (P < 0.001 and P < 0.01 respectively) and plasma renin activity (P < 0.001) in both control and FR50 rats. Under these conditions, FR50 rats exhibited lower (P < 0.05) plasma levels of ANP and higher (P < 0.05) plasma levels of aldosterone than controls (Table 2).

**Hypothalamic VP expression**

Under basal conditions, perinatal maternal food restriction was unable to affect significantly the hypothalamic VP gene expression in the PVN as well as in the SON (Table 2).

There was a significant perinatal regimen × condition interaction on VP gene expression in both PVN (F1,32 = 10.93, P = 0.002) and SON (F1,32 = 5.35, P = 0.027). This interaction was due to a significant (P < 0.05) increase in VP mRNA levels in controls but not in FR50 rats after 72 h dehydration (Table 2).

Table 2 Hypothalamic PVN and SON VP mRNA, plasma concentrations of VP, ANP and aldosterone, and PRA under basal conditions and after 72 h dehydration in 4-month-old male rats from mothers fed ad libitum (control, C) or subjected to a 50% food restriction during the last week of gestation and during lactation (FR50). Values are means ± S.E.M. (n = 9 animals per group).

<table>
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<tr>
<th>Experimental groups</th>
<th>Basal conditions</th>
<th>After 72 h dehydration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VP mRNA (OD/mm²)</td>
<td>VP (pg/ml)</td>
</tr>
<tr>
<td>C</td>
<td>88.36 ± 3.97</td>
<td>23.79 ± 1.98</td>
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<tr>
<td>FR50</td>
<td>78.95 ± 4.91</td>
<td>30.65 ± 3.64</td>
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<td></td>
<td>96.95 ± 1.17</td>
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<tr>
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<td>94.81 ± 1.90</td>
<td>1.92 ± 0.26</td>
</tr>
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*P < 0.05, **P < 0.01 FR50 vs respective control; ***P < 0.001 after dehydration values vs respective basal values.

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Density (B_max) and affinity (K_d) of ANP receptor subtypes

**In renal glomeruli** Under basal conditions, B_max of total ANP receptors as well as B_max of ANP-B and ANP-C receptors in renal glomeruli were not significantly altered by perinatal food restriction (Fig. 1A). No significant effect of perinatal malnutrition on the affinity (K_d) of either total ANP or ANP-B receptors was observed (data not shown).

A significant interaction between perinatal regimen and condition was found on B_max of both total ANP and ANP-B receptors (F_1,20 = 5.27, P = 0.033 and F_1,20 = 5.63, P = 0.028 respectively). In control rats exposed to 72 h water deprivation, B_max of total ANP receptors was not significantly affected. However, B_max of ANP-B receptors was significantly (P < 0.05) decreased while B_max of ANP-C receptors was significantly (P < 0.001) increased (Fig. 1A). Water-deprived FR50 rats exhibited increased (P < 0.01) B_max of total glomerular ANP binding sites. This rise was mainly due to increased (P < 0.001) B_max of ANP-C receptors whereas B_max of ANP-B receptors was not affected (Fig. 1A). The K_d values of total ANP receptors in renal glomeruli were not significantly affected by water deprivation in either controls or FR50 rats (data not shown). However, there was a significant perinatal regimen × condition interaction (F_1,20 = 4.93, P = 0.038) on the K_d values of ANP-B receptors. After 72 h dehydration, these K_d values were significantly (P < 0.05) reduced in controls (753.59±93.99 pmol/l after dehydration (n = 6) versus 1612.26±357.53 pmol/l under basal conditions (n = 6)) but not in FR50 rats (1257.19±101.40 pmol/l after dehydration (n = 6) versus 1332.04±188.54 pmol/l under basal conditions (n = 6)). Under these conditions, the K_d values of ANP-B receptors were higher (P < 0.01) in FR50 rats than in controls.

**In adrenal zona glomerulosa** Under basal conditions, perinatal maternal food restriction was unable to affect significantly the B_max of total ANP receptors in adrenal zona glomerulosa membranes in adult male offspring. In contrast, B_max of ANP-B receptors was significantly (P < 0.01) reduced

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**Figure 1** ANP receptor subtypes in kidneys, under basal conditions and after 72 h dehydration in 4-month-old male rats from mothers fed ad libitum (control, C) or subjected to a 50% food restriction during the last week of gestation and during lactation (FR50). (A) Density (B_max) of total ANP, ANP-B and ANP-C receptors in isolated renal glomeruli (n = 6 experiments per group). (B) Densitometric quantification of the hybridization signal for ANP-B_A and ANP-C receptor mRNAs normalized to GAPDH mRNA signal (n = 6 animals per group). Data are means±s.e.m. *P < 0.05, **P < 0.01 FR50 compared with respective control. (+) P < 0.05, (+++) P < 0.001 after dehydration values compared with respective basal values. Prot, protein.
whereas $B_{\text{max}}$ of ANP-C receptors was significantly ($P < 0.001$) increased in FR50 rats (Fig. 2A). On the other hand, perinatal malnutrition resulted in a significant decrease in the $K_d$ values of total ANP ($586.83 \pm 107.29$ pmol/l for FR50 rats ($n = 6$) versus $967.53 \pm 114.52$ pmol/l for controls ($n = 6$); $P < 0.05$) and ANP-B receptors ($382.79 \pm 57.40$ pmol/l for FR50 rats ($n = 6$) versus $769.93 \pm 67.88$ pmol/l for controls ($n = 6$); $P < 0.01$).

Water deprivation for 72 h was unable to significantly affect $B_{\text{max}}$ of total ANP receptors in adrenal zona glomerulosa membranes in either controls or FR50 rats (Fig. 2A). Nevertheless, a significant interaction between perinatal regimen and condition was found on $B_{\text{max}}$ of both ANP-B ($F_{1,20} = 23.44$, $P < 0.0001$) and ANP-C ($F_{1,20} = 66.05$, $P < 0.0001$) receptors. Thus, whereas dehydrated control rats showed a significant ($P < 0.05$) decrease in $B_{\text{max}}$ of ANP-B receptors and a significant ($P < 0.01$) increase in $B_{\text{max}}$ of ANP-C receptors, opposite effects were induced by water deprivation in FR50 rats. Indeed, dehydrated FR50 rats exhibited a significant ($P < 0.01$) rise in $B_{\text{max}}$ of ANP-B receptors and a significant ($P < 0.05$) decrease in $B_{\text{max}}$ of ANP-C receptors (Fig. 2A). The $K_d$ values of total ANP receptors in adrenal zona glomerulosa membranes were not significantly affected by water deprivation in either controls or FR50 rats (data not shown). However, there was a significant perinatal regimen $\times$ condition interaction ($F_{1,20} = 5.98$, $P = 0.024$) on the $K_d$ values of ANP-B receptors. This interaction was due to a significant ($P < 0.01$) decrease in these $K_d$ values in dehydrated controls ($486.53 \pm 54.57$ pmol/l after dehydration ($n = 6$) versus $769.93 \pm 67.88$ pmol/l under basal conditions ($n = 6$)) but not in water-deprived FR50 rats (data not shown).

**Expression of ANP receptor subtypes**

In kidneys Under basal conditions, perinatal maternal food restriction had no significant effect on ANP-B$_A$ receptor mRNA levels in kidneys but caused significant ($P < 0.01$) increases in ANP-C receptor mRNA levels (Figs 1B and 3).

Water deprivation for 72 h was unable to significantly affect mRNA levels of either ANP-B$_A$ or ANP-C receptors in controls and FR50 rats (Figs 1B and 3).
In adrenals

Under basal conditions, ANP-B\textsubscript{A} receptor mRNA levels in adrenals were not significantly affected in FR50 rats (Figs 2B and 3). In contrast, ANP-C receptor mRNA levels were significantly (P < 0.05) higher in FR50 rats than in controls (Figs 2B and 3).

Water deprivation for 72 h was unable to significantly affect ANP-B\textsubscript{A} receptor mRNA levels in either control or FR50 rats (Figs 2B and 3). Nevertheless, a significant interaction between perinatal regimen and condition (F\textsubscript{1,20} = 76.61, P < 0.0001) was found on ANP-C receptor gene expression. Indeed, ANP-C receptor mRNA levels were significantly (P < 0.01) increased in controls but significantly (P < 0.05) decreased in FR50 rats, following water deprivation (Figs 2B and 3).

Discussion

Basal conditions

Our study demonstrates that at 4 months of age, perinatally malnourished male rats showed somatic growth retardation. This confirms previous data in rats obtained in our laboratory, in which body weight was studied at weaning (40) or at 4 months of age (52). Thus, limited nutritional intake during the last week of gestation and lactation leads to persistent failure to catch up in body weight in the adult rat offspring, despite nutritional rehabilitation for several months. This indicates the strong influence of dietary restriction during the perinatal period and supports the earlier findings of Garofano et al. (53). Failure to catch up growth in perinatally malnourished rats could be linked to changes in the growth hormone (GH)/insulin-like growth factor-I (IGF-I) axis. IUGR children with a persistent growth failure have a decreased GH secretion based on impaired GH pulse amplitudes, as well as decreased IGF-I levels (54). After intrauterine malnutrition, juvenile and adult rats have significantly increased levels of somatostatin mRNA in the hypothalamic periventricular nucleus (55).

Four-month-old FR50 rats showed reduced absolute weight of kidneys. This is in accordance with the findings of Garofano et al. (53) who demonstrated a reduced absolute weight of kidneys in rat offspring from food restricted mothers, at 3 and 12 months of age. This long-lasting impairment is in keeping with the irreversible deficit in glomeruli described in rats subjected to a 50% intrauterine food restriction (28, 29). FR50 rats showed reduced absolute adrenal weight but increased relative adrenal weight. The latter results could reflect the differential somatic and adrenal growth in FR50 rats. The increase in relative adrenal weight in FR50 rats could be due to slower body than adrenal growth.

Under basal conditions, FR50 rats showed high circulating levels of VP and aldosterone without significant changes in plasma ANP concentrations and plasma renin activity. As VP mRNA in both hypothalamic PVN and SON was not significantly affected by perinatal maternal undernutrition, one can speculate that the release of VP rather than its synthesis was increased. The consequence of such high plasma VP levels could be an increase in water reabsorption in the kidney, which could contribute to the decrease in haematocrit observed in FR50 rats. The increase in circulating levels of aldosterone showed in FR50 rats agrees with the enhanced plasma aldosterone levels observed at four and eight weeks of age in rat offspring of protein-restricted mothers from gestational day 12 until delivery (24). In response to the increased plasma aldosterone levels in FR50 rats, reabsorption of sodium would be enhanced which could contribute to the relatively normal plasma osmolality. In FR50 rats, plasma osmolality remained relatively constant with a small and non significant increase of 6%. Plasma VP is regulated physiologically by changes in plasma osmolality and in blood volume or pressure (56). Osmoregulation is so precise that only 1% increase in plasma osmolality could stimulate VP release (56). So, the small and insignificant increase...
in plasma osmolality could, at least in part, justify the marked increase in plasma VP in FR50 rats.

Several factors might be involved in the increase in plasma aldosterone levels observed in FR50 rats. High plasma VP levels could have a role. VP has been shown to stimulate aldosterone secretion *in vivo* or in freshly isolated and in cultured rat adrenal glomerulosa cells (see review in 57). Moreover, the findings of Mazzocchi et al. (58) indicate that endogenous VP may be specifically involved in the maintenance of the growth and steroidogenic capacity of rat adrenal zona glomerulosa. In fact, VP receptors are located in the rat adrenal zona glomerulosa as reported by Guillou et al. (59). On the other hand, it is well known that the renin–angiotensin system is involved in the stimulation of aldosterone secretion (60). Our results showed that plasma renin activity was not significantly affected by perinatal maternal food restriction. Consistent with our findings, others have reported no significant differences in the plasma renin activity in adult offspring of rats fed a protein-restricted diet during pregnancy, compared with controls (22, 61). However, our results disagree with a study showing a decrease in plasma renin activity at four and eight weeks of age in rat offspring from protein-restricted mothers during the second half of pregnancy (24). Nevertheless, it is not possible to rule out that impairment of other components of the renin–angiotensin system by perinatal malnutrition in the rat offspring (24) suggest that increased plasma aldosterone levels may play a role in the development of hypertension in adult rat offspring from protein-restricted mothers. Thus, in our study, the long-lasting effects of perinatal maternal food restriction on plasma VP and aldosterone concentrations could account for the hypertensinogenic effects of perinatal malnutrition in the rat observed in a number of studies (22, 24).

**Responsiveness to water deprivation**

**In control rats** Our results showed that after 72 h water deprivation there was a significant decrease in body weight in control rats. Although changes in body mass are assumed to be due to changes in body fluid compartments, a portion of the weight lost by water-deprived animals may also be due to a reduction in food intake. In our study, food intake was not measured but other studies have reported that dehydrated rats ate significantly less food (35, 64, 65). Reduction of food intake during water deprivation reflects a homeostatic mechanism that minimizes the negative consequences of dehydration (66). Indeed, in dehydrated rats the intake of a conspicuous amount of dry food might further compromise osmolality and body fluid balance (67, 68).

In the present study, enhanced plasma osmolality induced by water deprivation resulted in a significant increase in plasma VP levels, which is a known response to dehydration (34, 35, 69). The increased circulating levels of VP in dehydrated control rats may be due to enhanced VP release and synthesis since VP mRNA levels were increased in both hypothalamic PVN and SON after 72 h dehydration. Indeed, there is other evidence that VP mRNA is increased after chronic stimuli such as water deprivation (34, 36, 69). Furthermore, the vasopressinergic response induced by volume reduction of different origins (e.g. haemorrhage, dehydration, etc) depends generally on the A1 noradrenergic cell group of the caudal ventrolateral medulla which provides a direct excitatory input to neurosecretory VP cells (70). Indeed, in rats, El Fazaa et al. (35) have demonstrated an increase in A1 noradrenergic activity after dehydration which probably contributes to the increase in VP release.

In agreement with the literature (38, 39), water deprivation in control rats resulted in a marked decrease in plasma ANP levels. The observed increases in haematocrit and plasma osmolality in these rats are signs of a reduced plasma volume which could lead to a...
In FR50 rats Our results demonstrated that after 72 h dehydration, FR50 rats showed increased plasma osmolality, hematocrit, plasma renin activity and circulating levels of aldosterone but decreased plasma ANP concentrations similar to controls. However, under these conditions, FR50 rats showed significantly higher plasma osmolality and plasma aldosterone levels but lower plasma ANP levels than controls. Thus, the endocrine response of FR50 rats to dehydration was qualitatively comparable to controls. Only quantitative differences were observed between FR50 and control rats with dehydration.

Plasma VP concentrations tended to rise in FR50 rats after dehydration, even though this increase was not statistically significant probably because of the individual variability in the FR50 group under basal conditions. Nevertheless, with dehydration the rise in plasma concentration of VP was slight in FR50 rats compared with controls (1.7-fold vs 31.7-fold). Since FR50 rats had high VP levels under basal conditions, they may not have been able to increase these much further in response to dehydration. Moreover, unlike controls, water-deprived FR50 rats did not have significant increases in VP gene expression in both hypothalamic PVN and SON. This could also have led to the lack of a rise in VP in response to dehydration in these rats.

In renal glomeruli, following 72 h dehydration, FR50 rats upregulated ANP-C receptors as did controls; however they were unable to downregulate ANP-B receptors which remained unchanged. In adrenal zona glomerulosa, dehydration in FR50 rats caused changes in the density of ANP-B and ANP-C receptors that were opposite to those seen in controls. Indeed, the FR50 rats upregulated ANP-B receptors whereas they downregulated ANP-C receptors after 72 h dehydration. Thus, dehydration-induced regulation of ANP receptors was altered in FR50 rats.

The expression of ANP receptors in water-deprived FR50 rats was similar to controls except for the high level of ANP-C receptor gene expression in adrenals. Indeed, after 72 h dehydration, ANP-C receptor gene expression in adrenals decreased in FR50 rats whereas it increased in controls. This could suggest disturbances in control of expression of this gene and we can only speculate about the mechanisms responsible for such alterations. Perinatal malnutrition might possibly induce factors involved in the transcriptional repression of this gene, which would only be activated in dehydration conditions. This possibility is consistent with the presence of cis-acting negative elements situated in three regions (from −1178 to −708, from −707 to −625 and from −248 to −145) of the mouse ANP-C receptor gene promoter (76). The decrease in ANP-C receptor mRNA could contribute to the decrease in adrenal ANP-C receptor density observed in dehydrated FR50 rats if the changes in mRNA directly translate into changes in receptor protein.

In conclusion, our study shows that perinatal maternal food restriction, under basal conditions, has long-term consequences on the endocrine regulation of the electrolyte and fluid balance, with mainly a significant rise in circulating levels of VP and aldosterone. This is associated with a decrease in ANP-B receptor density and an increase in ANP-C receptor density in adrenals and a rise in ANP-C receptor gene expression in both adrenals and kidneys. Perinatal maternal food restriction affects also the responsiveness to water
deprivation. Indeed, the rise in hypothalamic VP gene expression observed in controls is lacking in FR50 rats. In kidneys, FR50 rats are unable to downregulate the ANP-B receptors. In adrenals, they show opposite variations to controls in ANP-B receptor density and in both ANP-C receptor density and gene expression. These results emphasize the importance of maternal nutrition, especially during late gestation and lactation, its long-term influence on VP and aldosterone secretion and ANP binding sites of offspring and its implication in metabolic disorders that may lead to hypertension.

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