Both high and low maternal salt intake in pregnancy alter kidney development in the offspring

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THERE IS GROWING CONSENSUS (4, 44), that customary levels of salt intake in Western societies make a major contribution to elevated blood pressure (20, 28). Several mechanisms have been proposed to explain the effect of salt on blood pressure (21, 25, 27, 40). Excessive salt intake causes secession of endogenous cardiotoxic steroids, e.g., marinobufagenin (MBG), which induces plasmalemal Na+/K+-ATPase (6). The resulting effects include natriuresis, elevation of blood pressure, vasoconstriction, and heart and kidney fibrosis (6). High MBG serum concentrations in preeclamptic mothers are correlated with low birth weight (42). Furthermore, maternal pre-eclampsia is associated with higher blood pressure in the offspring (23).

In humans (31, 32, 34) indicate that a low nephron number is associated with higher blood pressure. In addition, prenatal programming has been shown to be related to factors such as obesity, insulin resistance, and other cardiovascular risk factors (8). Maternal diet is a modifiable factor influencing the offspring’s health in adult life. In experimental studies, several maternal factors, such as protein, calorie, and calcium deficiency are associated with a higher risk of the offspring developing hypertension; in humans, this association is less clear (12). In rats, exposure of dams to a low-protein diet at any period of pregnancy results in hypertension (37) and lower nephron number (63) in their offspring. Maternal high salt intake also influences organ development in the fetus, for instance, the heart (18).

Kidney development is orchestrated by a sequence of interplaying factors. Their disruption causes abnormal renal development. Fibroblast growth factor (FGF)-2 is produced in the ureteric bud; it prevents apoptosis of the metanephrogenic mesenchyme and induces formation of nephrons (17). Furthermore, expression of paired box transcription factor (Pax)-2 is required for the early phase of mesenchyme-to-epithelium transition and is subsequently downregulated as the tissue becomes more differentiated (55). Pax-2 knockout mice do not develop kidneys (61). Wilms’ tumor inhibitory protein 1 (WT-1) gene is expressed starting with the early stages of nephrogenesis. In contrast to Pax-2, it is expressed in glomeruli of fetal kidneys at a later stage than PAX genes (19). The expression of WT-1 increases as glomeruli mature and suppresses Pax-2 expression (56). Glial cell line-derived neurotrophic factor (GDNF) signaling is vital for kidney development, and GDNF knockout mice demonstrate kidney agenesis (57). The loss of one allele for GDNF causes a reduction in nephron number and adult hypertension (15). GDNF’s effects in kidney development are opposed by an inhibitor, sprouty-1 (9). In the absence of GDNF signaling, FGF-10 starts playing a major role in ureteric bud branching (46). Blockade of the renin-angiotensin system (RAS) during kidney development results in irreversible abnormalities in renal histology, including low nephron number (24, 65). Vascular endothelial growth factor (VEGF) is produced by differentiating podocytes and triggers formation of capillaries in the glomeruli (35).

Low-calorie and low-protein diets in dams cause low birth weight, low nephron number, and hypertension in their offspring (29, 64). Increased maternal salt intake has been asso-
associated with kidney dysfunction in the offspring (7, 45). Increased blood pressure has been reported in offspring after maternal high salt intake in one study (45), but only an elevated poststress blood pressure response has been observed by others (53). On the other hand, salt restriction in dams lead to lower birth weight (39). This topic is definitely of interest because it has been shown that maternal sodium intake determines blood pressure in the next generation (14).

In modern times, salt intake in humans has become excessive. There is, however, no knowledge of how far the salt restriction should go. It was the purpose of the present investigation to examine the effects of sodium intake below or above sodium intake generally used in rat experiments compared with standard sodium intake in dams on nephron formation and blood pressure in the offspring. We hypothesized that either below-usual or above-usual sodium intake in dams would reduce the number of glomeruli and increase blood pressure in the offspring. It was a further object of the study to identify potential pathways correlated with the changes in nephron numbers.

MATERIALS AND METHODS

Animals

All animals were handled according to written approval from the local authority for animal experiments (Regierungspraesidium Karlsruhe). Pregnant Sprague-Dawley rats were obtained from Charles River (Sulzfeld, Germany) at day 1 after conception. The animals were randomized to receive a diet based on a standard rodent diet (Ssniff, Soest, Germany) with modified sodium content: 0.07% (low sodium; LS; n = 34 dams); 0.51% (intermediate sodium; IS; n = 34 dams); or 3.0% (high sodium; HS; n = 35 dams) from the first day of pregnancy until weaning. The litters were standardized to identical size (n = 10/litter) by randomly culling the pups at birth with a 1:1 male-to-female ratio retained. Subsequently, one randomly chosen offspring per litter was used for each of the analyses. Offspring were separated from their mothers at the age of 4 wk and subsequently received the IS diet (0.51% Na). All animals were housed at a constant room temperature (21 ± 1°C) and humidity (75 ± 5%) and were exposed to a 12:12-h light-dark cycle. The animals had free access to deionized water and food. Body weight, food, and water consumption were monitored weekly.

ELISA

Urine was sampled for 24 h in metabolic cages at postnatal months 3, 6, and 9 (n = 10 male and 10 female offspring/group) at baseline and after 7 days of the HS (8%) diet. Urinary albumin excretion was measured with a rat-specific ELISA kit (50).

MBG was measured in samples of amniotic fluid taken from individual fetuses (n = 12/group) at gestation day 21 following extraction with C18 columns as described previously (54). ELISA plates were coated with a MBG-BSA conjugate at a dose of 5 ng/well. Anti-MBG monoclonal antibody (4G4, titer 1:1,000) derived from a mouse was employed with a MBG-BSA conjugate at a dose of 5 ng/well. Anti-MBG monovalent-horseradish peroxidase conjugate (Abcam, Cambridge, UK).

Kidney samples obtained on gestation day 21 (from 10 males and 10 females/group) and postnatal week 1 (from 10 males and 10 females/group) were homogenized. The protein concentration was determined by the Bradford method (Bio-Rad). Protein (25 μg) was electrophoresed on SDS-polyacrylamide gels. Subsequently proteins were electroblotted onto polyvinylidifluoride membranes (Immobilon-P, Millipore). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.5% Tween 20 (TBS-T) and incubated for 2 h at room temperature with the primary antibody against angiotensin-converting enzyme (ACE), FGF-2 (Chemicon), ANG II type 1 receptor (AT1R), renin, GDNF (Abcam), ANG II type 2 (AT2R) receptor, Pax-2, VEGF, WT-1, FGF-10, sprouty-1, and Na-K-ATPase subunits α1 and α3 (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) were used. Peroxidase labeling was detected using a chemiluminescence kit according to the manufacturer’s recommendations (GE fixation was performed using 4% phosphate-buffered formaldehyde at body temperature for morphological or ice-cold NaCl for molecular investigations, respectively. Tissue samples were prepared as previously described (50).

Blood Analyses

Blood samples were collected from the abdominal aorta at the time of death. The concentrations of sodium and potassium were determined in whole blood by an ionometer (Ionometer 2, Fresenius Medical Care). Serum parameters were analyzed using standard laboratory methods, and creatinine by Jaffe’s method.

Systolic Blood Pressure Measurement

In the male offspring, blood pressure was measured by telemetry from postnatal months 2–9 (n = 6/group) as previously described (49). Briefly, at the age of 8 wk, under isoflurane (Baxter) anesthesia a telemetering sensor (model PA-C40; Data Science International) was inserted into the abdominal aorta below the renal arteries, and the transmitter was fixed intraperitoneally.

Morphological Investigations

Glomerular stereology. All investigations were performed by an observer who was unaware of the study groups.

The number of glomeruli was estimated using the fractionator method (48). Each kidney (from 7–9 males and 7–9 females/group at each age) was dehydrated in graded ethanol, embedded in glycolmethacrylate (Technovit 7100; Heraeus Kulzer, Wehrheim, Germany), and cut exhaustively in 20-μm-thick sections. Every 30th section and its adjacent section (9–11 section pairs) were selected, mounted on one slide, and stained with periodic acid-Schiff. Counting was performed using an Olympus BX-50 microscope at a magnification of ×113 with an automated Märzhäuser Multi Control 2000 specimen stage (Märzhäuser, Wetzlar-Steindorf, Germany) and a digital camera (Pixelink PL-A686C) connected to a computer with newCAST software (Vysipharm, Horsholm, Denmark) to superimpose the counting frame. The glomeruli were counted if they were present inside the two-dimensional unbiased counting frame in one section (the sampling frame) but not in the adjacent section plane (the look-up section) and vice versa. The average volume of glomeruli was estimated as a ratio between the volume fraction of glomeruli estimated by point counting and the numerical density of glomeruli estimated by dissector-sampling as described above.

In 1-wk-old animals S-shaped bodies, immature, and mature glomeruli, defined according to Larsen (38), were counted separately. Developing glomeruli were organized in layers of different maturity (38). The number of layers was counted under ×400 magnification in several view-fields, depending on kidney size.

Western Blotting

Kidney samples obtained on gestation day 21 (from 10 males and 10 females/group) and postnatal week 1 (from 10 males and 10 females/group) were homogenized. The protein concentration was determined by the Bradford method (Bio-Rad). Protein (25 μg) was electrophoresed on SDS-polyacrylamide gels. Subsequently proteins were electroblotted onto polyvinylidifluoride membranes (Immobilon-P, Millipore). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.5% Tween 20 (TBS-T) and incubated for 2 h at room temperature with the primary antibody against angiotensin-converting enzyme (ACE), FGF-2 (Chemicon), ANG II type 1 receptor (AT1R), renin, GDNF (Abcam), ANG II type 2 (AT2R) receptor, Pax-2, VEGF, WT-1, FGF-10, sprouty-1, and Na-K-ATPase subunits α1 and α3 (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) were used. Peroxidase labeling was detected using a chemiluminescence kit according to the manufacturer’s recommendations (GE.
Healthcare). To control for variations in protein loading or transfer, membranes were washed and reincubated with anti-β-actin antibody (Abcam). The specific bands were quantified using computer software (ImageJ, National Institutes of Health).

**Immunohistochemistry**

Kidney samples obtained at postnatal week 1 (from 10 males and 10 females/group) were embedded in paraffin, and 2-µm sections were used for immune staining using primary antibodies against FGF-2 (Chemicon), angiotensin II type 1 receptor, GDNF (Abcam), angiotensin II type 2 receptor, FGF-10, and sprouty-1 (Santa Cruz Biotechnology). Biotin-labeled secondary antibodies, streptavidin-conjugated alkaline phosphatase (BioGenex) and Fast Red substrate (Dako) were used for visualization. Stained sections were analyzed by an observer unaware of the study groups. Negative controls were performed by omitting the primary antibody. Before the primary antibody was applied, endogenous biotin was blocked by 0.05% avidin in phosphate buffered saline followed by 0.005% biotin to saturate the bound avidin. All antibodies were tested to cross-react with rat samples.

**Real-Time PCR**

Total RNA was isolated from whole kidneys from offspring at gestation day 21 (10 males and 10 females/group) and postnatal week 1 (10 males and 10 females/group) using the SV Total RNA Isolation System (Promega) according to the manufacturer’s instructions, and real-time PCR was performed as previously described (50). Using primers for WT-1 (gcagagcaaccacggcac), Pax-2 (gactttaagagatgtgtcggagg), renin (ctgccgttgaggattcacaacc), ACE (gtggctacgagcatgacatca), AT1 receptor (tgatccccgtaagttgataat), α1-Na-K-ATPase (gggacg-cagttgcagacaaa), and α2-Na-K-ATPase (cgacagaggaaggactggt). Every sample was quantified using a gene-specific standard curve, and the average value of three different PCR runs normalized to GAPDH expression was taken for statistical evaluation.

**Statistical Analysis**

Data are given as means ± SD. For Western blotting, the intermediate-sodium group served as a reference, and the mean value of individual measurements was set as 100%. The value for each animal was expressed as the manifold of the reference. One randomly chosen offspring per litter was used for each of the analyses. Two-way (diet and sex) ANOVA was used, followed by Duncan’s multiple-range test for differences between groups. The results were considered significant when P was <0.05.

**RESULTS**

**Dams**

There was no difference between the groups with respect to body weight at the beginning and at end of pregnancy or with respect to weight gain during pregnancy (Table 1). Daily food consumption was significantly higher in dams on the HS compared with dams on IS and LS diets.

**Table 1. Body weight and food intake of dams**

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight, g</th>
<th>Gain in pregnancy</th>
<th>Food Intake During Pregnancy, g/day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At start of pregnancy</td>
<td>At end of pregnancy</td>
<td></td>
</tr>
<tr>
<td>Low sodium</td>
<td>205 ± 29</td>
<td>305 ± 29</td>
<td>100 ± 27</td>
</tr>
<tr>
<td>Intermediate</td>
<td>201 ± 29</td>
<td>298 ± 29</td>
<td>97 ± 22</td>
</tr>
<tr>
<td>High sodium</td>
<td>203 ± 32</td>
<td>294 ± 32</td>
<td>91 ± 26</td>
</tr>
<tr>
<td>ANOVA</td>
<td>NS</td>
<td>NS</td>
<td>26.6 ± 3.6*†</td>
</tr>
</tbody>
</table>

Values are means ± SD. NS, not significant. *P < 0.05 vs. low sodium. †P < 0.05 vs. intermediate sodium.

**Offspring Data: Litter Size, Body Weight, and Kidney Weight**

There was no significant difference in mean litter size between dams on the LS (12.5 ± 2.6 offspring), IS (13.5 ± 1.6 offspring), and HS diet (12.7 ± 1.6 offspring). Similarly, no difference between the groups was seen with respect to placenta weight (LS: 0.435 ± 0.056 g; IS: 0.432 ± 0.073 g; HS: 0.455 ± 0.059 g).

The body weight of offspring was not different between the groups examined at fetal day 21 and later, until postnatal week 12 (Table 2).

Kidney weight-to-body weight ratio were not different between the groups of offspring (Table 2). There was no difference in kidney weight-to-body weight ratio between male and female offspring.

At 12 wk of age, serum concentrations of sodium and potassium were not significantly different between the groups (Table 3). Serum concentrations of creatinine were significantly lower and creatinine clearances were higher in both male and female offspring of dams on a HS compared with a LS or IS diet (Table 3).

**Systolic Blood Pressure and Albuminuria**

Until postnatal month 4, there was no difference in mean arterial blood pressure between the groups of male offspring. Beginning at postnatal month 5 until the end of observation at month 9, mean arterial pressure was significantly higher in male offspring of dams on a LS or HS compared with a female offspring of dams on IS (Fig. 1C). At 3 mo of age, 24-h baseline urinary albumin excretion was significantly (P < 0.05, ANOVA) higher in the male offspring of dams on LS and HS compared with offspring of dams on IS, and the difference increased from month 6 (Fig. 1B). There was no difference in baseline albuminuria in female offspring (Fig. 1D). After 7 days of a HS diet, urinary albumin excretion increased more in male offspring of dams on LS and HS compared with offspring of dams on IS (Fig. 1D). In females, albuminuria increased on a HS diet in offspring of dams on LS from month 6 and in offspring of dams on HS on month 9 (Fig. 1E).

**Number of Glomeruli**

At 1 wk of age, the total number of glomeruli was significantly (P < 0.001, ANOVA) lower in offspring of dams on LS (23,900 ± 4,000) and even more in offspring of dams on HS (11,200 ± 1,800) compared with IS (30,800 ± 4,000). No difference was found between males and females (Fig. 2).

At 1 wk of age, the relative proportion (% of all glomeruli) of S-shaped bodies was significantly lower in offspring of
Table 2. **Body and organ weight in offspring**

<table>
<thead>
<tr>
<th>Group</th>
<th>21-Day Fetuses</th>
<th>1 wk</th>
<th>12 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body weight, g</td>
<td>Kidney weight, mg</td>
<td>Kidney weight/body weight ratio, mg/g</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low sodium</td>
<td>2.33 ± 0.14</td>
<td>6.55 ± 0.89</td>
<td>4.11 ± 0.51</td>
</tr>
<tr>
<td>Intermediate</td>
<td>2.37 ± 0.21</td>
<td>5.46 ± 1.23*</td>
<td>3.46 ± 0.65</td>
</tr>
<tr>
<td>High sodium</td>
<td>2.47 ± 0.18</td>
<td>7.32 ± 0.77†</td>
<td>4.01 ± 0.46</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low sodium</td>
<td>2.41 ± 0.19</td>
<td>6.25 ± 0.90</td>
<td>3.99 ± 0.52</td>
</tr>
<tr>
<td>Intermediate</td>
<td>2.45 ± 0.23</td>
<td>5.99 ± 0.15*</td>
<td>3.60 ± 0.34</td>
</tr>
<tr>
<td>High sodium</td>
<td>2.55 ± 0.19</td>
<td>7.38 ± 0.90†</td>
<td>3.95 ± 0.66</td>
</tr>
</tbody>
</table>

2-Way ANOVA

Sex NS NS NS NS NS NS
Salt intake of dams NS P < 0.001 NS NS NS NS P < 0.001 NS

Values are means ± SD. *P < 0.05 vs. low sodium †P < 0.05 vs. intermediate sodium.

dams on HS (4.3 ± 3.1%; 480 ± 350/kidney) compared with offspring of dams on LS (9.8 ± 6.3%; 3,020 ± 1,940/kidney) or IS (9.4 ± 3.8%; 2,250 ± 910/kidney) (Fig. 2).

The relative proportion of immature glomeruli was significantly different between the groups (Fig. 2).

The relative proportion of mature glomeruli was significantly higher in offspring of dams on HS (11.9 ± 7.1%; 1,330 ± 800/kidney) compared with offspring of dams on LS (6.0 ± 4.3%; 1,430 ± 1,030/kidney). The proportion of mature glomeruli in offspring of dams on HS was 8.3 ± 5.4%; 2,560 ± 1,670/kidney was not different from offspring of dams on HS and on LS (Fig. 2).

The number of layers of developing glomeruli was significantly (P < 0.001) higher in offspring of dams on HS (7.1 ± 0.6) compared with offspring of dams on LS (5.9 ± 0.9) and IS (5.8 ± 1.1). There was no significant difference in the number of glomeruli and their maturation between male and female offspring.

The average glomerular volume was significantly (P < 0.001) higher in offspring of dams on HS (1.47 ± 0.37 × 10⁶ μm³) compared with offspring of dams on LS (0.59 ± 0.18 × 10⁶ μm³) and IS (0.49 ± 0.09 × 10⁶/μm³) with no difference between males and females.

At 12 wk of age, the total number of glomeruli per kidney was significantly lower in offspring of dams on LS (19,200 ± 3,200) and lowest in offspring of dams on HS (12,300 ± 2,500) compared with offspring of dams on IS (31,600 ± 4,300) (Fig. 2). No difference in the number of glomeruli between male and female offspring was observed.

The average glomerular volume was significantly higher in offspring of dams on HS (6.91 ± 2.72 × 10⁶ μm³) compared with offspring of dams on LS (2.25 ± 0.89 × 10⁶ μm³) and on LS (3.51 ± 1.26 × 10⁶ μm³) (Fig. 2).

**Growth and Transcription Factors**

At 1 wk of age, expression of Pax-2 was significantly lower in offspring of dams on HS compared with offspring of dams on LS and IS (Fig. 3).

The protein expression of WT-1 was significantly higher both at term and at 1 wk of age in offspring of dams on HS compared with offspring of dams on LS and IS (Fig. 3). The mRNA expression of WT-1 was also significantly (P < 0.05) higher in offspring of dams on HS (term: 2.70 ± 0.65, week 1: 1.00 ± 0.38) compared with offspring of dams on LS (2.15 ± 0.57 and 0.71 ± 0.31, respectively) and IS (2.29 ± 0.52 and 0.65 ± 0.47, respectively).

At term, the expression of VEGF was significantly lower and the expression of basic fibroblast growth factor (FGF-2) and lowest in offspring of dams on HS (12,300 ± 2,500) compared with offspring of dams on IS (31,600 ± 4,300) (Fig. 2). No difference in the number of glomeruli between male and female offspring was observed.

The average glomerular volume was significantly higher in offspring of dams on HS (6.91 ± 2.72 × 10⁶ μm³) compared with offspring of dams on IS (2.25 ± 0.89 × 10⁶ μm³) and on LS (3.51 ± 1.26 × 10⁶ μm³) (Fig. 2).

**Table 3. Blood analyses in offspring at week 12**

<table>
<thead>
<tr>
<th>Group</th>
<th>Na⁺, mmol/l</th>
<th>K⁺, mmol/l</th>
<th>Creatinine, μmol/l</th>
<th>Creatinine clearance, ml/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low sodium</td>
<td>141 ± 3</td>
<td>3.9 ± 0.2</td>
<td>34.1 ± 8.4</td>
<td>1.53 ± 0.98</td>
</tr>
<tr>
<td>Intermediate</td>
<td>141 ± 2</td>
<td>3.9 ± 0.2</td>
<td>34.4 ± 9.4</td>
<td>1.35 ± 0.55</td>
</tr>
<tr>
<td>High sodium</td>
<td>140 ± 2</td>
<td>4.0 ± 0.3</td>
<td>29.4 ± 6.4†</td>
<td>2.37 ± 1.00†</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low sodium</td>
<td>141 ± 3</td>
<td>3.9 ± 0.2</td>
<td>29.1 ± 5.3</td>
<td>2.45 ± 1.07</td>
</tr>
<tr>
<td>Intermediate</td>
<td>140 ± 2</td>
<td>3.9 ± 0.2</td>
<td>30.6 ± 4.1</td>
<td>2.57 ± 0.59</td>
</tr>
<tr>
<td>High sodium</td>
<td>140 ± 2</td>
<td>3.8 ± 0.4</td>
<td>24.1 ± 3.7†</td>
<td>3.61 ± 1.39†</td>
</tr>
</tbody>
</table>

2-Way ANOVA

Sex NS NS P < 0.001 P < 0.001
Salt intake of dams NS NS P < 0.001 P < 0.001

Values are means ± SD. *P < 0.05 vs. low sodium †P < 0.05 vs. intermediate sodium.
higher in offspring of dams on HS compared with offspring of dams on IS (Fig. 3). At 1 wk of age, the expression of VEGF and FGF-2 was lower in kidneys of dams on HS compared with offspring of dams on LS and IS. Strong staining for FGF-2 was detected in proximal and distal tubuli, and weak staining was observed in glomeruli without differences in localization between groups.

The expression of glial cell line-derived neurotrophic factor (GDNF) was not different between the groups at term, but at week 1 it was higher in offspring of dams on HS and LS compared with offspring of dams on IS (Fig. 3). Staining for GDNF was detected in glomerular, proximal, and distal tubular compartments without differences in localization between groups.

At term, the expression of sprouty-1 was similar in all groups and was significantly higher at week 1 in both offspring of dams on HS and LS compared with IS (Fig. 3). Staining for sprouty-1 was strong in proximal tubuli, weak in distal tubuli, and absent in glomeruli. There was no difference in localization of staining between groups. At 1 wk of age, the sprouty-1/GDNF ratio was significantly higher in offspring of dams on HS (3.8 ± 1.0) and LS (2.3 ± 0.4) compared with IS (0.5 ± 0.2).

The expression of FGF-10 was lowest in offspring of dams on LS both at term and at 1 wk (Fig. 3). Staining for FGF-10 was strong in proximal tubuli, weak in distal tubuli, and absent in glomeruli. There was no difference in localization of staining between groups.

**Components of the RAS**

The protein expression of renin at term in offspring of dams on HS was significantly lower compared with offspring of dams on LS and IS (Fig. 4). Similar results were observed for mRNA: HS: 0.85 ± 0.28, *P* < 0.005 vs. LS: 1.04 ± 0.14, IS: 1.20 ± 0.27. At 1 wk of age, the expression of renin and ACE
was significantly higher in the offspring of dams on HS compared with offspring of dams on LS and IS.

At term, the protein expression of AT1R was significantly lower in offspring of dams on HS compared with offspring of dams on IS and LS (Fig. 4). This was confirmed at mRNA level: HS: 1.61 ± 0.34, P < 0.005 vs. IS: 2.16 ± 0.48, LS: 1.92 ± 0.45. At 1 wk of age, the expression of AT1R was highest in offspring of dams on HS, intermediate in offspring of dams on LS, and lowest in offspring of dams on IS. Staining for AT1R was detected in proximal and distal glomeruli but not in glomeruli in similar localization in all groups.

Both at term and at 1 wk of age, the expression of AT2R on the protein level was significantly lower in offspring of dams on HS and LS compared with offspring of dams on IS (Fig. 4). Staining for AT2R was detected in glomerular, proximal, and distal tubular compartments in similar localization in all groups.

**Sodium-Potassium Pump**

The expression of α1-Na\(^+\)-K\(^+\)-ATPase (at both protein and RNA levels) at term was significantly lower in offspring of dams on HS or LS compared with IS and, conversely, significantly higher in offspring of dams on HS or LS compared with IS at week 1 (Fig. 5). The expression of α3-Na\(^+\)-K\(^+\)-ATPase was significantly lower in offspring of dams on HS or LS compared with IS both at term and at week 1 (Fig. 5).

**MBG in Amniotic Fluid**

The concentration of MBG in the amniotic fluid was significantly higher in HS and LS compared with the IS group (Fig. 6).

**DISCUSSION**

The main finding of the present study is the identification of potential molecular mechanisms influencing nephron development in response to different salt intakes in dams as well as the documentation of diminished final nephron number in offspring of dams fed either a LS or HS diet during pregnancy and weaning. The lower prevalence of S-shaped bodies and the relative increase in mature glomeruli in the offspring of dams on HS may argue for accelerated maturation of glomeruli.

Our study does not answer the question of whether the reduced nephron number following modification of dietary salt was due to events in utero or during lactation. In contrast to humans, in rats nephrogenesis continues until 8 days postnatally. This was the reason we used the modified diet also during lactation. The issue has been raised whether HS causes regression of glomeruli; this possibility cannot be completely excluded, but no glomerular remnants were observed. The differences in food intake (and subsequently protein and calorie) by dams are unlikely to account for the lower nephron number observed in LS and HS groups, because food intake between LS and IS dams was not different and even higher in HS dams.

Blood pressure was higher in male offspring of dams on HS or LS diets after 5 mo of life. Similarly, blood pressure elevation has been shown in animals with a genetic deficit of glomeruli (15, 36) or after maternal protein restriction (66). Hypertension was also seen in humans with low nephron numbers (31, 32, 34). It is worth noting that previous studies have shown that, over time, blood pressure of animals with reduced nephron numbers becomes progressively more salt sensitive (47, 58). HS intake in pregnant rats caused a greater blood pressure response to stress (53) in their adult offspring and caused an elevation of resting blood pressure (62), which our study confirms. In contrast to the observation by Porter et al. (53), we observed elevation of baseline blood pressure in the male offspring of dams on HS, but the animals in the study by Porter were younger (2–6 mo) and the HS diet was continued in lactation and therefore until the end of nephrogenesis in this study.

![Fig. 2. A: number of glomeruli per kidney in female (○) and male (●) offspring at 1 wk of age. B: relative proportion of glomeruli at different stages of maturation at 1 wk of age. C and D: number and average volume, respectively, of glomeruli per kidney in female (○) and male (●) 12-wk-old offspring. There were fewer glomeruli in offspring of dams on LS or HS compared with IS both at 1 and at 12 wk of age. In addition, in offspring of dams on HS the glomeruli were bigger and the proportion of S-shaped bodies was lower.](http://ajprenal.physiology.org/)

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In the present study, maternal sodium intake in one group was 0.07%; this intake was chosen because more intense sodium restriction of the maternal diet (0.03%) causes growth restriction and later obesity, hypertension (10), and increased renin activity (43) in the offspring. Normal rat chow contains 0.3–0.5% sodium. This concentration would be excessive for humans, but is necessary for growing rodents. The HS diet (3% NaCl) represents a 5–10 times increase in sodium intake from the rodents baseline, similar to human increase in the industrial era (60). It was chosen to match the diets used in previous studies (18, 53, 62) and because this salt intake in pregnancy and lactation resulted in hypertension in the off-

Fig. 3. Expression of transcription and growth factors in offspring kidneys. Representative Western blots are shown. Values of the IS group were taken as reference (100%). By 2-way ANOVA, there was no difference between sexes. The expression of glial cell-derived neurotrophic factor (GDNF) and sprouty-1 was higher in LS and HS offspring at postnatal day 7. In HS offspring, the expression of Pax-2 and FGF-2 was lower while WT-1 was higher. In LS offspring, the expression of FGF-10 was lower. WT-1, Wilms' tumor-1.
spring rats (16). The diets were maintained during lactation to maintain the same salt intake until the end of nephrogenesis, i.e., until 8 days postnatally.

Brenner et al. (11) had proposed the hypothesis that bigger glomeruli are more susceptible to delayed loss of renal function, presumably as a result of glomerular hyperfiltration (59). The findings of higher creatinine clearance and bigger glomeruli in offspring of dams on HS are presumably associated with glomerular hyperfiltration. In a model of low glomerular numbers, i.e., offspring of rats of dams on a low-protein diet, administration of anti-Thy1 antibody caused more aggressive glomerulonephritis (51). An indirect indicator of the existence of a relationship between nephron number and progression of kidney disease in humans is the recent observation (26) that low birth weight (a surrogate marker for reduced nephron number) increases the risk of chronic kidney disease at adulthood.

It is of note that in the present study urinary albumin excretion was higher in the offspring of pregnant rats on both LS or HS, potentially a reflection of lower glomerular numbers. Our results further support the observation by Sanders et al. (58) of more severe albuminuria after salt loading in offspring with reduced nephron numbers. A relationship between growth restriction and albuminuria presumably exists in humans as well: Keijzer-Veen et al. (33) noted that individuals with low birth weight (a surrogate marker for reduced nephron number) increases the risk of chronic kidney disease at adulthood.

In the present study, the results were obtained by the design-based fractionator method (48). The final number of glomeruli in offspring of Sprague-Dawley dams on IS is in agreement with data reported by others (5, 30). In contrast to our results, Cardoso et al. (13) did not observe lower nephron number in offspring of dams with increased sodium intake in pregnancy. The discrepancy may be explained by a different sodium exposition, different rat strain, but also by a less-exact method to determine nephron number in the study of Cardoso et al. Interestingly, the increased albuminuria in those offspring was present in both studies.

We tried to identify factors potentially involved in the modulation of nephrogenesis by maternal salt intake. In the present study, offspring of pregnant rats on HS had not only lower nephron numbers but also lower renal expression of renin at birth. This finding is in agreement with observations in the low-protein model of intrauterine growth restriction (64). Similar to what was seen in offspring of dams on a low-protein diet (3), the expression of AT2R was reduced at term and on day 7 in offspring of dams on LS or HS as well. In this model, however, the expression of renin, AT1R, and ACE was higher 7 days after birth, possibly pointing to transplacental modulation of the RAS by dietary salt of the mother and potential partial escape from salt overload during nursing. The postnatal activation of the RAS may account for accelerated maturation of immature glomeruli in the HS group.

One weakness of the study is that the dams were not pair-fed, but no correlation was found between food intake of the dam and total number of glomeruli of the offspring.

Fig. 4. Expression of the renin-angiotensin system (RAS) components in offspring kidneys. Representative Western blots are shown. Values of the IS group were taken as reference (100%). By 2-way ANOVA, there was no difference between sexes; group differences are marked. In HS offspring, the expression of renin and the ANG II type 1 receptor (AT1R) was lower at term, but the expression of renin, ACE, and AT2R receptor was higher 1 wk postnatally compared with IS group. The expression of the AT2R was lower in both HS and LS compared with IS at term and at week 1.
The increased MBG levels in the amniotic fluid are most probably the result of increased placental synthesis. MBG has been identified in the placenta, and its increase in preeclampsia have been well documented (22). In our study in offspring of dams on HS or LS, the expression of \( \frac{\text{Na}}{\text{H}} \)-K\(-\text{ATPase} \) in fetal kidneys at term was decreased in parallel with the elevation of MBG resembling the decrease of plasmalemmal \( \frac{\text{Na}}{\text{H}} \)-K\(-\text{ATPase} \) observed in response to MBG in vitro (41). Furthermore, 7 days after birth the expression of \( \frac{\text{Na}}{\text{H}} \)-K\(-\text{ATPase} \) in the kidneys increased dramatically to values greatly above controls, suggesting that high MBG levels were no longer present. A causal role of MBG in regulating nephron number is likely and needs further study. This is of special interest in view of the findings that in preeclampsia blood pressure was lowered by scavenging antibodies interfering with cardiotonic steroids (2) and children of preeclamptic mothers have higher blood pressure (23).

In line with the observation in Pax-2\(^{-/-}\) mice which have smaller kidneys (61), we observed downregulation of Pax-2 in the offspring of dams on HS, also consistent with the hypothesis that downregulation of Pax-2 by salt loading contributes to the cessation of the formation of new glomeruli.

In line with its inhibitory role for Pax-2 (56), we observed higher expression of WT-1 in the kidneys of the offspring of dams on HS accompanied by lower Pax-2 expression in this group. Moreover, the expression of WT-1 was increased earlier, i.e., gestation day 21, and Pax-2 was reduced at a later stage.

Contrary to what could be anticipated from studies in GDNF-deficient mice (15), we did not observe suppression of GDNF, which is known to support nephronogenesis; in fact, its expression was higher in groups with lower nephron number. Most importantly, we demonstrated that the expression of sprouty-1, an endogenous inhibitor of GDNF signaling during kidney development (9), was increased even more, probably causing a relative GDNF deficiency leading to nephron deficit, further supporting the causal role of the balance between sprouty-1 and GDNF for optimal nephron number (9).

It is of note that in fetal life several different pathways apparently contributed to the same net effect, low nephron...
number. The upregulation of WT-1 and downregulation of Pax-2 is in agreement with the observation in the low-protein model (1), but our findings of elevated GDNF and lower FGF-2 are opposite of the findings in that model (1).

In view of the ability of FGF-2 to induce nephronogenesis (52), we observed that less FGF-2 expression was paralleled by lower numbers of newly formed nephrons in the kidneys of offspring of dams on HS, potentially consistent with a causal role of FGF-2.

We demonstrated lower FGF-10 expression in offspring of dams on LS accompanying lower number of glomeruli. This observation is of potential relevance because removal of FGF-10 in the setting of lack of GDNF signaling stops kidney development (46).

We were able to demonstrate that an intervention in one parameter (sodium intake in dams) resulted in modification of different molecular pathways depending whether the primary signal was “increased” (HS intake caused FGF-2 deficiency) or “decreased” (LS intake caused FGF-10 deficiency). In summary, the low nephron number in the offspring of LS, accompanying lower number of glomeruli. This is most probably due to lower FGF-10 expression.

The reduced expression of VEGF in kidneys of offspring exposed to HS is probably explained by the reduced number of glomeruli as VEGF is secreted by maturing podocytes at a late stage of kidney development (35). Taken together, the above findings indicate that both too low and too high maternal salt intakes retard development of new glomeruli, resulting in a nephron deficit. An intervention in maternal diet translated into altered protein expression in the kidneys of the offspring extending beyond development in utero. High maternal salt intake was further accompanied by accelerated maturation of glomeruli, glomerulomegaly, and albuminuria in the offspring. If the findings in the rat can be extrapolated to humans, both too low and too high salt intake during pregnancy would be a risk factor for hypertension and renal damage in the offspring.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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