Postnatal Time Frame for Renal Vulnerability to Enalapril in Rats

GREGOR GURON,* NIELS MARCUSSEN,‡ ANNNIKA NILSSON,* BIRGITTA SUNDELIN,† and PETER FRIBERG*

*Department of Physiology, Institute of Physiology and Pharmacology, Göteborg University and †Department of Pathology, Karolinska Hospital, Stockholm, Sweden; and ‡Department of Pathology, Aarhus Kommune Hospital, University of Aarhus, Denmark.

Abstract. Angiotensin-converting enzyme inhibition or angiotensin II type I receptor blockade in neonatal rat, but not in weaned, rats induces irreversible renal histologic abnormalities and an impaired urinary concentrating ability. The aim of the present study was to define the postnatal time frame when the rat kidney is vulnerable to an interruption of the renin-angiotensin system. Male Wistar rats received daily injections of enalapril (10 mg/kg, intraperitoneally) during different age intervals within 3 to 24 d of age. Fluid handling and urinary concentrating ability, renal function under pentobarbital anesthesia, and kidney histology using stereologic techniques were evaluated in adult rats. Enalapril treatment within 3 to 13 d after birth induced abnormalities in renal function and morphology long-term, whereas treatment initiated at 14 d of age did not. The main histologic alterations were papillary atrophy, and a reduction in the volume of tubular epithelial cells in association with an increase in the proportion of interstitium, throughout the cortex and outer medulla. Functionally, the predominant defect was an impairment in urinary concentrating ability, which correlated with the degree of papillary atrophy. In conclusion, the vulnerable age interval for the induction of irreversible renal abnormalities by enalapril was the first 13 d after birth in the rat. This postnatal time span coincides with the completion of nephrogenesis and a period of marked tubular growth and differentiation, suggesting a pivotal role for angiotensin II in these processes.

All components of the renin-angiotensin system (RAS) are expressed in the immature kidney and are developmentally regulated in a tissue-specific manner, with increased gene expression and elevated renal angiotensin II (AngII) content occurring perinatally compared with the adult (1,2). Although the physiologic role of the RAS in the developing kidney remains to be elucidated, the spatio-temporal pattern of the expression of AngII receptors (3–5), together with in vitro evidence showing trophic and proliferative effects of AngII on different renal cell types (6), has suggested that AngII may be involved in the regulation of renal growth and differentiation. In support of this notion, angiotensin-converting enzyme (ACE) inhibition or AngII type I (AT1) receptor antagonism has been shown to inhibit renal growth (7) and induce renal histopathologic abnormalities (7–9) in the neonatal rat. Confirmation of an essential role for the RAS in renal development has recently been provided by gene-targeting studies, in which mice deficient in ACE (10,11) or angiotensinogen (12,13) developed alterations in renal histology similar to those observed after neonatal pharmacologic blockade of the RAS in rats (8,9,14–17).

We have demonstrated previously that neonatal ACE inhibition or AT1 receptor antagonism, during the first 24 d of life in rats (8,14–17), and in pigs (18), induces irreversible abnormalities in renal morphology characterized by papillary atrophy, interstitial inflammation and fibrosis, tubular atrophy, and renal vascular changes, which were accompanied by an impaired urinary concentrating ability. It is unknown exactly what postnatal time interval the rat kidney is vulnerable to an interruption of the RAS. However, when treatment with the AT1 receptor antagonist losartan was begun in weanling rats of 3 wk of age, no renal histologic abnormalities were reported (19), indicating that the vulnerable time frame is restricted to the preweaning period. Illustrating the importance of AT1 receptor stimulation during the first 10 to 12 d after birth, AngII blockade during this early period produces renal histopathologic alterations resembling those we have reported (7,20). Still, the consequences of inhibiting the RAS later during the preweaning period have not been systematically investigated. Addressing this issue may be important when viewed from a clinical perspective. The use of ACE inhibitors during pregnancy has been shown to be associated with renal tubular dysplasia in the neonate (21,22), suggesting that the RAS, in addition to its role in regulating perinatal renal function, may be important in mediating normal renal morphogenesis, similar to the situation in rodents and pigs. Thus, from this viewpoint it is of considerable interest to determine at what
stage in development pharmacologic blockade of the RAS can be initiated without inducing renal abnormalities.

The aim of the present study was to define the postnatal time interval during the preweaning period when the rat kidney is dependent on an intact RAS for normal development. For this purpose, the ACE inhibitor enalapril was administered during different time intervals from day 3 to day 24 after birth, and renal function and histology were evaluated in adulthood.

Materials and Methods

General Procedures

Time-mated, female Wistar rats (Charles River UK Ltd., Margate, Kent, United Kingdom) were transported to our facility on the 16th day of pregnancy and carefully observed for determination of the day of delivery. Gender was determined in 2-d-old pups, and males were included in the study. Weight-matched male pups were divided into seven groups (n = 8 per group) receiving daily intraperitoneal injections of enalapril (10 mg/kg) or isotonic saline vehicle in equivalent volumes of 10 ml/kg, during different time intervals neonatally. Enalapril was administered during the following age intervals: from 3 to 9 (E3–9); 10 to 16 (E10–16); 17 to 24 (E17–24); 3 to 13 (E3–13); 14 to 24 (E14–24), and 3 to 24 (E3–24), days of age. Controls received vehicle from 3 to 24 d of age. During the age interval 3 to 24 d, all rats were injected with vehicle on days when enalapril was not administered. Rats had free access to normal rat chow and tap water and were kept in rooms with a controlled temperature of 24°C and a 12:12 dark/light cycle (6 p.m. to 6 a.m.) throughout the study. All experiments were approved by the regional ethics committee in Göteborg.

Protocol

Baseline measurements of fluid handling were performed at 7, 9, and 11 wk of age (n = 7 per group). Maximal urine osmolality (Uosmmax) was assessed in 11-wk-old rats (n = 7 per group). Renal function in pentobarbital anesthetized rats was analyzed in 12- to 14-wk old rats (n = 8 per group). After clearance experiments, rats were sacrificed and renal histology was investigated by stereologic methods (n = 8 per group).

Fluid Handling and Maximal Urine Osmolality

Rats were kept individually in metabolic cages with free access to powdered rat chow (Na+: 120 mmol/kg; K+, 153 mmol/kg) and tap water. After 2 d of acclimatization, baseline measurements were performed during a 24-h period (6 p.m. to 6 p.m.). Food and water intake, urine volume, and body weight were measured daily. Urine was collected in preweighed vials under mineral oil and analyzed for intake, urine volume, and body weight were measured daily. Urine was collected in preweighed vials, and urine density was assumed to be 1.00 g per ml urine. Mean arterial BP (MAP) and heart rate were recorded continuously with Statham pressure transducers connected to a Grass-polygraph.

Filtration fraction (FF, %) was calculated as (GFR/ERPF) × 100. Tubular solute-free water reabsorption (TmH2O) was calculated as osmolar clearance (Cosm) − V, where Cosm = [Uosm/plasma osmolality (Posm)/V × V = urine flow rate. Fractional urinary excretion rates of sodium (FENa%, %) and potassium (FEPK, %) were estimated as the ratio of their respective clearances to that of 51Cr-EDTA, taken as GFR × 100. Data are presented as mean values for the three clearance periods.

Kidney Histology

General Procedures for Stereologic Investigations. After renal clearance experiments, kidneys were rapidly excised, decapsulated, weighed, and immersion-fixed in 4% formaldehyde in phosphate-buffered saline, pH 7.4. Using unbiased stereologic methods, the following estimations were made by an investigator blinded to treatment groups: (1) volumes of the different zones in the kidney, i.e., cortex, outer stripe of the outer medulla (OSOM), inner stripe of the outer medulla (ISOM), and inner medulla, where the zonal definition determined by Kriz and Bankir (23) was used; (2) volumes of glomerular tufts, tubular epithelium, and interstitium in the cortex and OSOM; and (3) volumes of tubular epithelium and interstitium in the ISOM.

Left kidneys were cut in slices with a thickness of 2 mm, using a device with parallel razor blades (24). The cutting was done at a 90° angle to the longitudinal axis of the kidney. The 2-mm-thick slices were embedded in paraffin and cut into 3-μm-thick serial sections, which were stained with hematoxylin and eosin, periodic acid-Schiff (PAS), and Masson’s trichrome. Every 2-mm-thick slice was investigated, meaning that approximately five to seven from each kidney were examined. All morphometric analyses were performed on the same PAS-stained sections. Sections were placed in an Olympus microscope that was connected to a video camera and a computer. The computer generated a point set on the screen, and the video recorded the microscopic field. In systematic order with random start using a stage motor, sections were investigated by point counting, and the number of points hitting each structure was estimated and related to the total number of hits (24). From estimated volume fractions, absolute volumes of kidney structures were calculated assuming that the specific gravity of kidneys was 1 g/cm³.

Volume of Kidney Zones. Volumes of cortex, OSOM, ISOM, and inner medulla were estimated. A ×4 objective was used, and each

125I-hippuran (Institutt for Energiteknikk, Kjeller, Norway), respectively. Rats were anesthetized with pentobarbital (60 mg/kg, intraperitoneally) and tracheotomized with a polyethylene catheter (PE 240) to facilitate spontaneous breathing. Body temperature was maintained at 38°C throughout the experiment. The left jugular vein and carotid artery were catheterized with PE 50 tubing. The urinary bladder was catheterized through a midline abdominal incision by a PE 160 catheter. Throughout the experiment, rats were infused with 53Cr-EDTA (20 μCi/kg per h, intravenously), 125I-hippuran (10 μCi/kg per h, 125 μg/kg per h, intravenously), and pentobarbital (12 mg/kg per h, intra-arterially) dissolved in isotonic saline yielding a total infusion rate of 10 ml/kg per h. In a previous study (17), we have shown that renal hippuran extraction is unaltered in adult neonatally enalapril-treated rats when hippuran is administered in the dose used in the present study. After 45 min of equilibration, three consecutive 20-min urine collection periods with midpoint arterial blood sampling (0.3 ml) were performed. Urine was collected in preweighed vials, and urine density was assumed to be 1.00 g per ml urine. Mean arterial BP (MAP) and heart rate were recorded continuously with Statham pressure transducers connected to a Grass-polygraph.

Filteration fraction (FF, %) was calculated as (GFR/ERPF) × 100. Tubular solute-free water reabsorption (TmH2O) was calculated as osmolar clearance (Ccosm) − V, where Cosm = [Uosm/plasma osmolality (Posm)/V × V = urine flow rate. Fractional urinary excretion rates of sodium (FENa%, %) and potassium (FEPK, %) were estimated as the ratio of their respective clearances to that of 51Cr-EDTA, taken as GFR × 100. Data are presented as mean values for the three clearance periods.

Kidney Histology

General Procedures for Stereologic Investigations. After renal clearance experiments, kidneys were rapidly excised, decapsulated, weighed, and immersion-fixed in 4% formaldehyde in phosphate-buffered saline, pH 7.4. Using unbiased stereologic methods, the following estimations were made by an investigator blinded to treatment groups: (1) volumes of the different zones in the kidney, i.e., cortex, outer stripe of the outer medulla (OSOM), inner stripe of the outer medulla (ISOM), and inner medulla, where the zonal definition determined by Kriz and Bankir (23) was used; (2) volumes of glomerular tufts, tubular epithelium, and interstitium in the cortex and OSOM; and (3) volumes of tubular epithelium and interstitium in the ISOM.

Left kidneys were cut in slices with a thickness of 2 mm, using a device with parallel razor blades (24). The cutting was done at a 90° angle to the longitudinal axis of the kidney. The 2-mm-thick slices were embedded in paraffin and cut into 3-μm-thick serial sections, which were stained with hematoxylin and eosin, periodic acid-Schiff (PAS), and Masson’s trichrome. Every 2-mm-thick slice was investigated, meaning that approximately five to seven from each kidney were examined. All morphometric analyses were performed on the same PAS-stained sections. Sections were placed in an Olympus microscope that was connected to a video camera and a computer. The computer generated a point set on the screen, and the video recorded the microscopic field. In systematic order with random start using a stage motor, sections were investigated by point counting, and the number of points hitting each structure was estimated and related to the total number of hits (24). From estimated volume fractions, absolute volumes of kidney structures were calculated assuming that the specific gravity of kidneys was 1 g/cm³.

Volume of Kidney Zones. Volumes of cortex, OSOM, ISOM, and inner medulla were estimated. A ×4 objective was used, and each
field of vision included a grid with six points. The total number of points hitting each kidney ranged from 220 to 270.

**Volume of Structures in the Cortex and OSOM.** Volumes of glomerular tufts, proximal tubular cells, distal tubular cells (including tubular epithelium of the thick ascending limb of Henle [TAL], distal convoluted tubule, connecting tubule, and collecting duct [CD]), atrophic tubules, and interstitium (including interstitial capillaries) were estimated. A ×20 objective was used and each field of vision included a grid with 16 points. The total number of points hitting the cortex and OSOM ranged from 320 to 420 per kidney.

**Volume of Structures in the ISOM.** Volumes of TAL + CD cells, atrophic tubules, and interstitium (including interstitial capillaries and the thin descending and ascending limbs) were estimated. A ×40 objective was used, and the total number of points hitting the ISOM ranged from 240 to 320 per kidney.

**Analytical Methods**

Osmolality was determined by the method of freezing point depression (Wide Range Advanced Osmometer model 3MO; Advanced Instruments, Needham Heights, MA). Urine and plasma were analyzed for radioactivity and sodium and potassium concentrations using a Packard three-channel scintillation counter (model 5019; Packard Co., Amana, IA) and a flame spectrophotometer (model FLM; Radiometer, Copenhagen, Denmark), respectively.

**Statistical Analyses**

Data in text, tables, and figures are expressed as mean ± SEM. Differences between experimental groups were evaluated by one-way ANOVA or the nonparametric Kruskal–Wallis test, where appropriate, followed by modified unpaired t test or Mann–Whitney test to compare pairs of groups using the Bonferroni method to adjust for multiple comparisons. The following comparisons were performed between individual groups: each enalapril-treated group versus saline vehicle, and group E3–9 versus E10–16, E3–13, and E3–24. Body weights during neonatal treatment were analyzed with ANOVA for repeated measurements. The correlation between two continuous variables was evaluated by linear regression analysis. \( P < 0.05 \) was considered statistically significant. Analyses were performed using software StatView 4.1 for Macintosh (Abacus Concepts, Berkeley, CA).

**Results**

**Body Weights**

During the age interval 3 to 24 d, enalapril treatment produced reductions in body weight in groups E10–16 (\( P < 0.05 \) on days 19 to 24), E17–24 (\( P < 0.05 \) on days 21 to 24), E14–24 (\( P < 0.05 \) on days 17 to 24), and E3–24 (\( P < 0.05 \) on days 20 to 24) (data not shown). The effect of neonatal enalapril administration was transient as there were no differences between groups in body weight at an adult age.

**Fluid Handling and Maximal Urine Osmolality**

Mean values of repeated measurements of fluid handling at 7, 9, and 11 wk of age are summarized in Table 1. Groups E3–13 and E3–24 showed increases in water intake and \( V \), and a reduction in \( U \text{osm} \) compared with vehicle (Table 1). Similarly, group E3–9 had an increased water intake and a reduction in \( U \text{osm} \), even though \( V \) did not differ from controls (Table 1). Water intake and \( V \) were elevated and \( U \text{osm} \) was reduced in groups E3–13 and E3–24 compared with group E3–9 (Table 1). \( U \text{osm}_{\text{max}} \), measured after 30 h of water deprivation and following dDAVP administration, was reduced in groups E3–9, E10–16, E3–13, and E3–24 (Table 1, Figure 1). Moreover, \( U \text{osm}_{\text{max}} \) was decreased in group E3–24 compared with group E3–9 (Table 1, Figure 1). Groups E17–24 and E14–24 did not differ from vehicle-treated rats in any of the investigated parameters (Table 1, Figure 1).

**Renal Function and Hemodynamics**

Body and wet kidney weights were similar in neonatally enalapril- and vehicle-treated rats at the time of renal clearance.

**Table 1.** Fluid handling, urinary electrolyte excretion, and urine osmolality\(^a\)

<table>
<thead>
<tr>
<th>Group</th>
<th>Water Intake (ml/kg per 24 h)</th>
<th>( V ) (ml/kg per 24 h)</th>
<th>( U \text{osm} ) (mosm/kg)</th>
<th>( U_{Na}V ) (mmol/kg per 24 h)</th>
<th>( U_{K}V ) (mmol/kg per 24 h)</th>
<th>( U \text{osm}_{\text{max}} ) (mosm/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>95 ± 5</td>
<td>38 ± 2</td>
<td>1678 ± 49</td>
<td>6.0 ± 0.4</td>
<td>10.4 ± 0.4</td>
<td>3366 ± 146</td>
</tr>
<tr>
<td>E3–9</td>
<td>118 ± 4(^b)</td>
<td>54 ± 4</td>
<td>1254 ± 78</td>
<td>6.8 ± 0.3</td>
<td>11.1 ± 0.2</td>
<td>2548 ± 118(^b)</td>
</tr>
<tr>
<td>E10–16</td>
<td>112 ± 8</td>
<td>47 ± 6</td>
<td>1420 ± 100</td>
<td>6.0 ± 0.2</td>
<td>9.7 ± 0.4</td>
<td>2658 ± 206(^b)</td>
</tr>
<tr>
<td>E17–24</td>
<td>96 ± 2</td>
<td>40 ± 2</td>
<td>1565 ± 86</td>
<td>6.1 ± 0.3</td>
<td>9.9 ± 0.3</td>
<td>3559 ± 122</td>
</tr>
<tr>
<td>E3–13</td>
<td>141 ± 6(^b,c)</td>
<td>76 ± 6(^b,c)</td>
<td>882 ± 63(^b,c)</td>
<td>6.4 ± 0.3</td>
<td>10.5 ± 0.4</td>
<td>1978 ± 120(^b)</td>
</tr>
<tr>
<td>E14–24</td>
<td>104 ± 3</td>
<td>41 ± 2</td>
<td>1556 ± 54</td>
<td>6.1 ± 0.3</td>
<td>10.4 ± 0.7</td>
<td>3331 ± 265</td>
</tr>
<tr>
<td>E3–24</td>
<td>164 ± 7(^b,c)</td>
<td>93 ± 7(^b,c)</td>
<td>774 ± 55(^b,c)</td>
<td>5.7 ± 0.3</td>
<td>9.3 ± 0.4</td>
<td>1562 ± 135(^b,c)</td>
</tr>
</tbody>
</table>

\( P \) Value\(^d\): \<0.05 <0.05 <0.05 NS NS <0.05

\(^a\) Mean values of repeated measurements of fluid handling at 7, 9, and 11 wk of age during baseline conditions. Maximal urine osmolality (\( U \text{osm}_{\text{max}} \)) was assessed in 11-wk-old rats after 30 to 36 h of water deprivation and dDAVP administration. Rats had been treated neonatally with enalapril (10 mg/kg per d) or isotonic saline vehicle during different age intervals from 3 to 24 d of age (\( n = 7 \) per group). Groups receiving enalapril (E) are denoted with the respective age interval during which the drug was administered. \( V \), urine flow rate; \( U \text{osm} \), urine osmolality; \( U_{Na}V \), urinary sodium excretion; \( U_{K}V \), urinary potassium excretion; dDAVP, desmopressin acetate. Values are mean ± SEM.

\(^b\) \( P < 0.05 \) versus vehicle.

\(^c\) \( P < 0.05 \) versus group E3–9.

\(^d\) ANOVA.
TcH2O were similar in neonatally enalapril- and vehicle-treated vehicle; † between groups were detected in FENa (Table 3). Cosm and group E3–13 compared with vehicle, whereas no differences rats both in absolute values and when corrected for GFR (data Table 2).

Renal function and hemodynamics in anesthetized rats a

Additionally, Uosm was decreased in groups E3–9 and V and a reduction in Uosm compared with vehicle (Table 3). MAP, ERPF, or GFR (Table 2). Similar to the situation in conscious rats, groups E3–13 and E3–24 showed an increased V and a reduction in Uosm compared with vehicle (Table 3). FEK was elevated in group E3–13 compared with vehicle, whereas no differences between groups were detected in FENa (Table 3). Cosm and TcH2O were similar in neonatally enalapril- and vehicle-treated rats both in absolute values and when corrected for GFR (data not shown). However, when TcH2O was expressed as a fraction of Cosm (an index of distal solute delivery), TcH2O/Cosm was decreased in groups E3–13 and E3–24 compared with vehicle (Table 3). Groups E17–24 and E14–24 did not differ from vehicle-treated rats in any of the parameters investigated during clearance experiments (Tables 2 and 3).

Figure 1. Bar graph shows maximal urine osmolality (Uosm max), expressed in percentage of vehicle, in 11-wk-old Wistar rats treated neonatally with enalapril (10 mg/kg per d, intraperitoneally) during different age intervals from 3 to 24 d of age (n = 7 per group). Uosm max was measured after 30 to 36 h of water deprivation and after desmopressin acetate (dDAVP) administration. Groups receiving enalapril (E) are denoted with the respective age interval during which the drug was administered. Values are mean ± SEM. ∗ P < 0.05 versus vehicle; † P < 0.05 versus group E3–9.

Kidney Histology

Volume of Kidney Zones. Morphometric analyses revealed no significant difference between groups in absolute volumes of the cortex or ISOM (Table 4). The volume of the OSOM was increased in group E3–24 compared with vehicle-treated rats (Table 4). Due to various degrees of papillary atrophy, the volume of the inner medulla was reduced in groups E3–9, E10–16, E3–13, and E3–24 (Table 4, Figure 2, A and B, Figure 3). The reduction in inner medullary volume was more pronounced in group E3–24, and less marked in group E10–16, when compared with group E3–9 (Table 4). Linear regression analysis demonstrated a significant, positive relationship between the inner medullary volume and Uosm max in rats treated neonatally with enalapril (y = 1324 + 35x, r = 0.82, P < 0.05) (Figure 4). The inner medulla appeared normal in groups E14–24 (Figure 3D) and E17–24.

Volume of Structures in the Cortex and OSOM. Reductions in volumes of proximal tubular cells, in association with increases in the proportion of interstitium, were demonstrated in groups E3–9, E10–16, E3–13, and E3–24 (Table 5). Increases in the interstitial volume were associated with focal areas of interstitial fibrosis, tubular dilation, and interstitial infiltration of mononuclear inflammatory cells (Figure 2, C and D). Atrophic tubules were detected in groups E3–9, E10–16, E3–13, and E3–24 (Table 5). There were no differences between groups in the volume of glomerular tufts or distal tubular cells (Table 5). Glomeruli did not show any morphologic abnormalities in neonatally enalapril-treated rats. Notably, interlobular arteries appeared abnormal with concentric wall thickening comprising both the intima and media in neonatally

Table 2. Renal function and hemodynamics in anesthetized rats a

<table>
<thead>
<tr>
<th>Group</th>
<th>BW (g)</th>
<th>KW wet (g/kg BW)</th>
<th>MAP (mmHg)</th>
<th>GFR (ml/min per g KW)</th>
<th>ERPF (ml/min per g KW)</th>
<th>FF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>413 ± 10</td>
<td>7.0 ± 0.2</td>
<td>121 ± 4</td>
<td>1.20 ± 0.07</td>
<td>4.26 ± 0.24</td>
<td>28 ± 2</td>
</tr>
<tr>
<td>E3–9</td>
<td>414 ± 10</td>
<td>7.0 ± 0.2</td>
<td>121 ± 5</td>
<td>1.10 ± 0.08</td>
<td>3.49 ± 0.22</td>
<td>31 ± 2</td>
</tr>
<tr>
<td>E10–16</td>
<td>413 ± 14</td>
<td>6.5 ± 0.2</td>
<td>124 ± 5</td>
<td>1.14 ± 0.07</td>
<td>3.84 ± 0.23</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>E17–24</td>
<td>409 ± 9</td>
<td>6.5 ± 0.3</td>
<td>122 ± 5</td>
<td>1.22 ± 0.11</td>
<td>4.20 ± 0.30</td>
<td>29 ± 2</td>
</tr>
<tr>
<td>E3–13</td>
<td>400 ± 12</td>
<td>6.6 ± 0.1</td>
<td>125 ± 4</td>
<td>1.13 ± 0.07</td>
<td>3.62 ± 0.20</td>
<td>31 ± 1</td>
</tr>
<tr>
<td>E14–24</td>
<td>406 ± 9</td>
<td>7.1 ± 0.2</td>
<td>125 ± 4</td>
<td>1.19 ± 0.05</td>
<td>3.87 ± 0.25</td>
<td>31 ± 2</td>
</tr>
<tr>
<td>E3–24</td>
<td>415 ± 18</td>
<td>6.8 ± 0.5</td>
<td>123 ± 7</td>
<td>1.12 ± 0.12</td>
<td>3.94 ± 0.32</td>
<td>28 ± 2</td>
</tr>
</tbody>
</table>

P Valueb NS NS NS NS NS

a Renal function and hemodynamics in 14-wk-old, pentobarbital anesthetized rats, treated neonatally with enalapril (10 mg/kg per d) or isotonic saline vehicle during different age intervals from 3 to 24 d of age (n = 8 per group). Groups receiving enalapril (E) are denoted with the respective age interval during which the drug was administered. BW, body weight; KW wet, wet kidney weight; MAP, mean arterial pressure; ERPF, effective renal plasma flow; FF, filtration fraction. Values are mean ± SEM.
b ANOVA.
Table 3. Renal electrolyte and water handling in anesthetized rats

<table>
<thead>
<tr>
<th>Group</th>
<th>V (µl/min per g KW)</th>
<th>Uosm (mosm/kg)</th>
<th>Posm (mosm/kg)</th>
<th>P Na (mmol/L)</th>
<th>P K (mmol/L)</th>
<th>FENa (%)</th>
<th>FEK (%)</th>
<th>T H2O/Cosm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>4.96 ± 0.78</td>
<td>1861 ± 147</td>
<td>292 ± 2</td>
<td>140 ± 2</td>
<td>3.9 ± 0.1</td>
<td>0.32 ± 0.13</td>
<td>24.7 ± 3.3</td>
<td>0.83 ± 0.02</td>
</tr>
<tr>
<td>E3–9</td>
<td>7.09 ± 1.59</td>
<td>1337 ± 122 b</td>
<td>302 ± 2</td>
<td>143 ± 1</td>
<td>3.9 ± 0.1</td>
<td>0.44 ± 0.17</td>
<td>27.9 ± 2.6</td>
<td>0.74 ± 0.03</td>
</tr>
<tr>
<td>E10–16</td>
<td>7.22 ± 1.11</td>
<td>1405 ± 121 b</td>
<td>302 ± 1</td>
<td>143 ± 1</td>
<td>3.8 ± 0.1</td>
<td>0.45 ± 0.20</td>
<td>34.0 ± 3.2</td>
<td>0.77 ± 0.03</td>
</tr>
<tr>
<td>E17–24</td>
<td>4.85 ± 0.60</td>
<td>1667 ± 122</td>
<td>299 ± 2</td>
<td>144 ± 1</td>
<td>4.0 ± 0.1</td>
<td>0.34 ± 0.09</td>
<td>27.0 ± 3.1</td>
<td>0.80 ± 0.02</td>
</tr>
<tr>
<td>E3–13</td>
<td>12.09 ± 2.03 b</td>
<td>1042 ± 86 b</td>
<td>300 ± 1</td>
<td>143 ± 2</td>
<td>3.7 ± 0.1</td>
<td>0.86 ± 0.34</td>
<td>37.6 ± 3.8 b</td>
<td>0.68 ± 0.03 b</td>
</tr>
<tr>
<td>E14–24</td>
<td>6.41 ± 0.83</td>
<td>1603 ± 74</td>
<td>298 ± 2</td>
<td>140 ± 1</td>
<td>3.6 ± 0.1</td>
<td>0.32 ± 0.06</td>
<td>28.1 ± 1.3</td>
<td>0.80 ± 0.01</td>
</tr>
<tr>
<td>E3–24</td>
<td>13.80 ± 3.65 b</td>
<td>909 ± 99 b</td>
<td>303 ± 2</td>
<td>143 ± 1</td>
<td>3.7 ± 0.1</td>
<td>0.42 ± 0.14</td>
<td>34.8 ± 2.7</td>
<td>0.64 ± 0.04 b</td>
</tr>
</tbody>
</table>

P Value<sub>c</sub> <0.05  NS NS NS NS <0.05  <0.05

<sup>a</sup> Renal electrolyte and water handling in 14-wk-old, pentobarbital anesthetized rats, treated neonatally with enalapril (10 mg/kg per d) or isotonic saline vehicle during different age intervals from 3 to 24 d of age (n = 8 per group). Groups receiving enalapril (E) are denoted with the respective age interval during which the drug was administered. Posm, plasma osmolality; P Na, plasma sodium concentration; P K, plasma potassium concentration; FENa, fractional urinary sodium excretion; FEK, fractional urinary potassium excretion; T H2O/Cosm, tubular free water reabsorption/osmolar clearance. Values are mean ± SEM.

<sup>b</sup> P <0.05 versus vehicle.

<sup>c</sup> ANOVA.

Table 4. Volume of kidney zones

<table>
<thead>
<tr>
<th>Group</th>
<th>V (cortex)</th>
<th>V (OSOM)</th>
<th>V (ISOM)</th>
<th>V (IM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>902 ± 26</td>
<td>366 ± 10</td>
<td>154 ± 4</td>
<td>62 ± 2</td>
</tr>
<tr>
<td>E3–9</td>
<td>888 ± 38</td>
<td>400 ± 17</td>
<td>152 ± 6</td>
<td>28 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>E10–16</td>
<td>851 ± 34</td>
<td>368 ± 15</td>
<td>148 ± 6</td>
<td>35 ± 1&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>E17–24</td>
<td>872 ± 31</td>
<td>339 ± 12</td>
<td>142 ± 5</td>
<td>59 ± 2</td>
</tr>
<tr>
<td>E3–13</td>
<td>818 ± 15</td>
<td>398 ± 7</td>
<td>154 ± 3</td>
<td>24 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>E14–24</td>
<td>900 ± 15</td>
<td>332 ± 5</td>
<td>155 ± 3</td>
<td>58 ± 1</td>
</tr>
<tr>
<td>E3–24</td>
<td>842 ± 24</td>
<td>452 ± 13b</td>
<td>155 ± 4</td>
<td>11 ± 1&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

P Value<sub>d</sub> <0.05  NS <0.05  NS<0.05

<sup>a</sup> Volumes (V) of cortex, outer stripe of outer medulla (OSOM), inner stripe of outer medulla (ISOM), and inner medulla (IM) were assessed using stereologic methods (see Materials and Methods). Measurements were performed on immersion-fixed kidneys from 14-wk-old rats treated neonatally with enalapril (10 mg/kg per d) or isotonic saline vehicle during different age intervals from 3 to 24 d of age (n = 8 per group). Groups receiving enalapril (E) are denoted with the respective age interval during which the drug was administered. Values are mean ± SEM (in mm<sup>3</sup>).

<sup>b</sup> P <0.05 versus vehicle.

<sup>c</sup> P <0.05 versus group E3–9.

<sup>d</sup> ANOVA.

Discussion

The main finding of the present study was that ACE inhibition within the first 13 d of age in the rat induced abnormalities in renal function and morphology long-term, whereas treatment initiated at 14 d of age did not. Moreover, within the vulnerable age interval, enalapril treatment of a duration of only 7 d was sufficient for producing irreversible renal abnormalities.

In accordance with previous studies (8,14–17), the main functional abnormality in adult rats treated neonatally with enalapril was an impairment in urinary concentrating ability. We have previously been able to demonstrate that this impairment in urine concentration is of renal origin and due to a specific defect in tubular free water reabsorption that may be explained by the atrophy of the papilla (17). In support of this notion, Uosm<sub>max</sub> after water deprivation and dDAVP administration was reduced in those groups showing papillary damage in the present study. Moreover, there was a strong correlation between the morphometrically assessed volume of the inner medulla and Uosm<sub>max</sub> in neonatally enalapril-treated rats. Results obtained in hydropenic rats during clearance experiments underscored that the long-term adverse effects of neonatal ACE inhibition on kidney function primarily seem to involve tubular water handling, as renal hemodynamic parameters and GFR were normal in neonatally enalapril-treated rats.

In accord with our previous observations (15,16), the rate of fractional urinary potassium excretion tended to be elevated in adult neonatally enalapril-treated rats, reaching a significant elevation in urine concentration is of renal origin and due to a specific defect in tubular free water reabsorption that may be explained by the atrophy of the papilla (17). In support of this notion, Uosm<sub>max</sub> after water deprivation and dDAVP administration was reduced in those groups showing papillary damage in the present study. Moreover, there was a strong correlation between the morphometrically assessed volume of the inner medulla and Uosm<sub>max</sub> in neonatally enalapril-treated rats. Results obtained in hydropenic rats during clearance experiments underscored that the long-term adverse effects of neonatal ACE inhibition on kidney function primarily seem to involve tubular water handling, as renal hemodynamic parameters and GFR were normal in neonatally enalapril-treated rats.

In accord with our previous observations (15,16), the rate of fractional urinary potassium excretion tended to be elevated in adult neonatally enalapril-treated rats, reaching a significant level in group E3–13. We hypothesize that this finding is due to the prevailing renal histologic changes and secondary to elevations in distal tubular flow rate and/or sodium delivery, which would enhance the rate of potassium secretion in principal cells.

enalapril-treated rats, mainly in groups E3–13 and E3–24 (Figure 2C). Groups E14–24 and E17–24 did not differ from vehicle-treated rats in any of the investigated parameters (Table 5).

Volume of Structures in the ISOM. The volume of TAL and CD epithelial cells was reduced, and the interstitial volume was increased, in groups E3–9, E10–16, E3–13, and E3–24 (Table 6, Figure 2, E and F). Atrophic tubules were detected in groups E10–16, E3–13, and E3–24 (Table 6). Similar to the pattern in the cortex and OSOM, increases in the interstitial volume of the ISOM were associated with interstitial fibrosis and inflammation, and tubular dilation (Figure 2, E and F). Groups E14–24 and E17–24 did not differ from vehicle-treated rats in any of the investigated parameters (Table 6).
Similar to the present study, Spence et al. (25) investigated the susceptible period of developmental toxicity for losartan in rats and found that the critical period for losartan-induced kidney lesions, which were remarkably similar to those reported here, was from embryonic day 15 to 20. The finding that maternal exposure to losartan postnatally did not induce renal

Figure 2. Kidney sections from 12- to 14-wk-old rats treated neonatally from 3 to 24 d of age with enalapril (10 mg/kg per d, intraperitoneally) (left column; A, C, and E) or isotonic saline vehicle (right column; B, D, and F). Panels show representative histology from the papilla (A and B), cortex (C and D), and inner stripe of the outer medulla (ISOM) (E and F). Neonatally enalapril-treated rat shows papillary atrophy, and dilation of collecting ducts (A). Histologic abnormalities in the cortex (C) and ISOM (E) are characterized by an increased proportion of interstitium and interstitial inflammation and fibrosis. Note wall thickening of interlobular artery in the cortex (C, arrow) and dilated tubules in the ISOM (E, asterisk) of neonatally enalapril-treated rat. Sections were stained with hematoxylin and eosin. Magnification: ×20 in A and B; ×90 in C through F.
lesions in the pups contrasts with observations in the present and previous studies in which neonates were directly injected with ACE inhibitors or losartan (7–9, 14–17, 20). This discrepancy is likely to be due to the fact that the concentration of losartan and its active metabolite is low in maternal rat milk (25). Importantly, enalapril treatment initiated after 14 d of age did not produce any renal abnormalities in the present study. Still, group E10–16 developed rather severe alterations in both kidney morphology and function, which presumably were a consequence of ACE inhibition on postnatal day 10 to 13. The present study, in conjunction with findings by Spence et al. (25), suggests that the developing rat kidney is vulnerable to an interruption of the RAS from embryonic day 15 to postnatal day 13.

One might argue that the age-dependent effect of neonatal enalapril treatment on kidney integrity could be attributed to differences in the clearance of the drug. We addressed this issue in a pilot study in which plasma and kidney enalaprilat (the active diacid form of enalapril) concentrations were analyzed in rats after drug administration from 3, 10, or 14 d of age. Notably, although enalaprilat concentrations tended to increase in younger rats, values in the oldest age group were high enough over 24 h to assume a nearly complete inhibition of ACE activity (G. Guron, unpublished observations). Thus, our finding of severe renal abnormalities in rats treated with enalapril from 3 or 10 d of age, but no renal lesions when drug administration was initiated at 14 d of age or later, is unlikely to be explained by differences between groups in the degree of ACE inhibition.

At embryonic day 15, both AT1 and AT2 receptors are expressed in the undifferentiated mesenchyme of the developing rat kidney, in which metanephrogenesis has been initiated and the first nephrons have been formed (4, 5). Characteristically, renal development will then continue in a centrifugal manner with more mature nephrons being localized deep in the forming kidney, while the superficial cortex will contain a nephrogenic zone with immature nephrons and undifferentiated mesenchyme. Nephrogenesis is completed in the second postnatal week in the rat (26–28), although tubular differentiation continues until the time of weaning (26–31) and the functional maturation even after that (31). Postnatally, the AT2 receptor is expressed throughout nephrogenesis but is undetectable in the kidney after postnatal day 14 (3, 5). On the contrary, AT1 receptors become more prevalent neonatally and localized to glomeruli and tubular structures of more mature nephrons, arteries, the medullary rays, and the outer medulla (3, 4). A striking observation in the present study was that rats treated with enalapril within postnatal day 3 to 13 showed a reduction in the volume of tubular epithelial cells, in association with an increased proportion of interstitium, throughout the cortex and outer medulla. One may speculate that this alteration could be due to an inhibition of tubular growth and maturation. This notion is supported by the tubular localization of AT1 receptors (3, 4), and observations of a decreased cell proliferation and inhibited nephron differentiation in neonatal rats subjected to ACE inhibition or AT1 receptor blockade (7, 20). Furthermore, tubules undergo marked maturational changes during the first 2 wk of life in the rat. Apart from the formation of new nephrons, continuing into the second week of life, loops of both superficial and juxtamedullary nephrons, collecting ducts, and vascular bundles elongate throughout the medulla during this period (27, 28, 30). At about 2 wk of age, the medulla is morphologically mature and the tubular epithelium of loops of Henle and collecting ducts has differentiated into the same structural appearance as in the adult kidney (26, 27, 29). When evaluating the clinical implications of results in the present study, one should keep in mind that while nephrogenesis continues into the second postnatal week in rats, this process has been completed in humans at 36 wk of gestation although tubular growth and differentiation proceed after birth (31, 32).

Clearly, the mechanisms whereby neonatal ACE inhibition induced irreversible renal lesions need further investigation. It is noteworthy that Tufro-McReddie et al. demonstrated that administration of losartan to neonatal rats, in addition to de-

![Figure 4](image-url) Scatterplot shows the correlation between the absolute volume of the inner medulla $V_{(IM)}$ and maximal urine osmolality (Uosm$_{\text{max}}$) in adult Wistar rats treated neonatally with enalapril (10 mg/kg per d, intraperitoneally) during different age intervals from 3 to 24 d of age. $V_{(IM)}$ was assessed using unbiased stereologic methods (see Materials and Methods). Uosm$_{\text{max}}$ was measured after 30 to 36 h of water deprivation and after dDAVP administration. Linear regression analysis revealed a significant, positive relationship between $V_{(IM)}$ and Uosm$_{\text{max}}$ ($P < 0.05$, $r = 0.82$, $n = 42$).
Table 5. Volume of structures in the cortex and outer stripe of the outer medulla

<table>
<thead>
<tr>
<th>Group</th>
<th>$V_{(PT)}$</th>
<th>$V_{(DT)}$</th>
<th>$V_{(INT)}$</th>
<th>$V_{(GLOM)}$</th>
<th>$V_{(ATUB)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>855 ± 44</td>
<td>120 ± 7</td>
<td>85 ± 6</td>
<td>31 ± 4</td>
<td>0</td>
</tr>
<tr>
<td>E3–9</td>
<td>661 ± 32$^b$</td>
<td>97 ± 8</td>
<td>175 ± 13$^b$</td>
<td>39 ± 5</td>
<td>0.7 ± 0.5</td>
</tr>
<tr>
<td>E10–16</td>
<td>658 ± 36$^b$</td>
<td>104 ± 12</td>
<td>162 ± 10$^b$</td>
<td>34 ± 5</td>
<td>1.3 ± 0.9</td>
</tr>
<tr>
<td>E17–24</td>
<td>769 ± 53</td>
<td>93 ± 10</td>
<td>73 ± 5</td>
<td>33 ± 4</td>
<td>0</td>
</tr>
<tr>
<td>E3–13</td>
<td>602 ± 37$^b$</td>
<td>90 ± 7</td>
<td>170 ± 7$^b$</td>
<td>27 ± 3</td>
<td>11.4 ± 4.4$^{b,c}$</td>
</tr>
<tr>
<td>E14–24</td>
<td>717 ± 30</td>
<td>106 ± 4</td>
<td>83 ± 3</td>
<td>32 ± 11</td>
<td>0</td>
</tr>
<tr>
<td>E3–24</td>
<td>589 ± 34$^b$</td>
<td>104 ± 9</td>
<td>190 ± 5$^b$</td>
<td>33 ± 3</td>
<td>9.4 ± 2.3$^{b,c}$</td>
</tr>
<tr>
<td>$P$ Value$^d$</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

$^a$ Volumes (V) of proximal tubular cells (PT), distal tubular cells (DT), interstitium (INT), glomerular tufts (GLOM), and atrophic tubules (ATUB) were assessed using stereologic methods (see Materials and Methods). Experiments were performed on immersion-fixed kidneys from 14-wk-old rats treated neonatally with enalapril (10 mg/kg per d) or isotonic saline vehicle during different age intervals from 3 to 24 d of age (n = 8 per group). Groups receiving enalapril (E) are denoted with the respective age interval during which the drug was administered. Values are mean ± SEM (in mm$^3$).

$^b$ P < 0.05 versus vehicle.

$^c$ P < 0.05 versus group E3–9.

$^d$ ANOVA.

Table 6. Volume of structures in the inner stripe of the outer medulla

<table>
<thead>
<tr>
<th>Group</th>
<th>$V_{(TAL + CD)}$</th>
<th>$V_{(INT)}$</th>
<th>$V_{(ATUB)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>86 ± 3</td>
<td>42 ± 2</td>
<td>0</td>
</tr>
<tr>
<td>E3–9</td>
<td>55 ± 3$^b$</td>
<td>64 ± 4$^b$</td>
<td>0</td>
</tr>
<tr>
<td>E10–16</td>
<td>57 ± 4$^b$</td>
<td>55 ± 3$^b$</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>E17–24</td>
<td>75 ± 4</td>
<td>42 ± 2</td>
<td>0</td>
</tr>
<tr>
<td>E3–13</td>
<td>57 ± 3$^b$</td>
<td>64 ± 3$^b$</td>
<td>0.7 ± 0.3$^b$</td>
</tr>
<tr>
<td>E14–24</td>
<td>75 ± 3</td>
<td>50 ± 1</td>
<td>0</td>
</tr>
<tr>
<td>E3–24</td>
<td>56 ± 3$^b$</td>
<td>66 ± 4$^b$</td>
<td>0.7 ± 0.3$^b$</td>
</tr>
<tr>
<td>$P$ Value$^c$</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

$^a$ Volumes (V) of thick ascending limb of Henle and collecting duct tubular epithelial cells (TAL + CD), interstitium (INT), and atrophic tubules (ATUB) were assessed using stereologic methods (see Materials and Methods). Experiments were performed on immersion-fixed kidneys from 14-wk-old rats treated neonatally with enalapril (10 mg/kg per d) or isotonic saline vehicle during different age intervals from 3 to 24 d of age (n = 8 per group). Groups receiving enalapril (E) are denoted with the respective age interval during which the drug was administered. Values are mean ± SEM (in mm$^3$).

$^b$ P < 0.05 versus vehicle.

$^c$ ANOVA.

laying nephron maturation, arrested renal vascular development (7). In support of a role for AngII in renal vascular development, McCausland et al. observed an interrupted descent of abnormal vasa recta bundles in the outer medulla of neonatally enalapril-treated rats (9). It is possible that these vascular abnormalities, in association with a reduction in renal perfusion pressure, may inhibit renal growth and maturation secondary to hypoperfusion. In this respect, the papilla may be particularly vulnerable as blood flow is low to this region of the kidney already at baseline. However, chronic neonatal administration of either hydralazine (7) or nifedipine (17) do not induce kidney lesions, indicating that a BP reduction per se does not account for the renal abnormalities observed in the present study. Interestingly, gene targeting of each of the known AngII receptors, i.e., the AT$_{1A}$ (33), AT$_{1B}$ (34) or AT$_2$ (35), does not induce renal abnormalities comparable to those observed in ACE- or angiotensinogen-deficient mice, or those reported in the present study. However, double mutant mice deficient in both the AT$_{1A}$ and AT$_{1B}$ receptor develop marked renal morphologic changes (36). This observation, together with findings in pharmacologic studies (7–9), clearly indicate that a lack of AT$_1$ receptor stimulation accounts for the renal lesions described in the present study. Regarding the etiology of papillary atrophy, Niimura et al. found a downregulation of platelet-derived growth factor-A mRNA in hypoplastic papillae of homozygous mice deficient in angiotensinogen (13), proposing that AngII may regulate the transcription of renal growth factors of possible importance for normal maturational growth of the papilla. Even though glomeruli appeared histologically normal in neonatally enalapril-treated rats in the present study, experiments were not designed to investigate the involvement of AngII in nephron induction and glomerulogenesis, and additional studies are needed to elucidate these issues.

Finally, in addition to the induction of renal lesions, we have persistently been able to show that ACE inhibition during the first 3 wk after birth in the rat produces a transient attenuation of somatic growth that becomes evident during the third week of life (8,14). Notably, in the present study all enalapril-treated groups, except groups E3–9 and E3–13, showed transient reductions in body weight during the end of the third and/or beginning of the fourth week of age. This finding indicates that neonatal enalapril treatment has an inhibitory effect on somatic growth, which is dissociated from the induction of renal lesions, and that the growth attenuation is primarily associated with enalapril administration after 2 wk of age.

In conclusion, the vulnerable age interval for the induction of irreversible renal abnormalities by enalapril was the first
13 d after birth in the rat. This postnatal time span coincides with the completion of nephrogenesis and a period of marked tubular growth and differentiation, suggesting a pivotal role for AngII in these processes. The results of the present study underscore that ACE inhibitors or AT1 receptor antagonists should be avoided in pregnant women, and used with great care in patients in whom nephrogenesis and/or tubulogenesis have not been completed.

Acknowledgments
This study was supported by the Göteborg Medical Society, the Swedish Medical Society, and the Swedish Medical Research Council (Grants 9047 and 11133). The authors thank Merck, Sharp & Dohme (Sollentuna, Sweden) for providing enalapril maleate.

References
31. Edwards BR, Mendel DB, LaRochelle FT Jr, Stern P, Valtin H: Postnatal development of urinary concentrating ability in rats:


