Angiotensin-converting enzyme inhibition aggravates renal interstitial injury resulting from partial unilateral ureteral obstruction in the neonatal rat

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Chen CO, Park MH, Forbes MS, Thornhill BA, Kiley SC, Yoo KH, Chevalier RL. Angiotensin-converting enzyme inhibition aggravates renal interstitial injury resulting from partial unilateral ureteral obstruction in the neonatal rat. Am J Physiol Renal Physiol 292: F946–F955, 2007. First published November 14, 2006; doi:10.1152/ajprenal.00287.2006.—Congenital urinary tract obstruction is the most important cause of renal insufficiency in infants and children, and angiotensin-converting enzyme (ACE) inhibitors attenuate the progression of renal disease in adults. ACE inhibitors are increasingly utilized in children with progressive renal disease. Because angiotensin is necessary for normal renal development, we examined the effects of ACE inhibition both during and immediately following the period of postnatal nephrogenesis in the neonatal rat subjected to sham operation or partial unilateral ureteral obstruction (UUO) under general anesthesia within the first 48 h of life. Rats in group I received enalapril 30 mg/kg body wt (or vehicle) daily for the first 10 days, while in group II, the 10 days of treatment began 10 days after surgery. Kidneys were harvested at day 21 and analyzed for apoptosis (TUNEL), interstitial macrophages (ED-1 immunohistochemistry), myofibroblasts (α-smooth muscle actin), and collagen (Sirius red). Partial UUO delayed glomerular maturation and increased ipsilateral renal macrophage infiltration, α-smooth muscle actin and Sirius red staining. In group I, enalapril increased myofibroblast accumulation in sham-operated kidneys, but not in obstructed kidneys. In contrast, in group II, enalapril further increased macrophage, myofibroblast, and collagen accumulation following partial UO. The relative abundance of components of the kallikrein-kinin system, measured by Western blot, was not altered by partial UUO in the 14- and 28-day-old rat. Thus, in contrast to its salutary effects at later ages, ACE inhibition can worsen injury to the partially obstructed kidney during renal maturation even after the completion of nephrogenesis.

OBSTRUCTIVE NEPHROPATHY REMAINS the major cause of renal insufficiency in infants and children (51). Although the lesion develops in fetal life, there is progression of the injury throughout postnatal life, often despite surgical relief of obstruction. This finding has led to attempts to attenuate the progression of renal injury.

The activity of the intrarenal renin-angiotensin system (RAS) is increased during early development and is further enhanced by ureteral obstruction. Angiotensin plays an important regulatory role in renal development, affecting the renal expression of important growth factors, such as transforming growth factor-β1 (58). Interference with the RAS in the developing kidney leads to a broad spectrum of renal maldevelopment, including thickened vasculature, abnormal glomeruli, tubular dilatation, and increased accumulation of extracellular matrix (22). Mutations of components of the RAS can result in major alterations in renal organogenesis, including the development of hydropneumohrosis and spontaneous ureteral obstruction (45). This may be a consequence of maldevelopment of the renal pelvis, leading to defective ureteral peristalsis and functional obstruction (41).

Paradoxically, chronic overactivity of the RAS resulting from persistent UUO actually contributes to increased renal production of transforming growth factor-β1 and interstitial fibrosis in neonatal or adult rats (10, 11, 29). Thus, inhibition of angiotensin has been shown to attenuate many forms of renal injury and to preserve renal function in renal disease (47). Specifically, angiotensin-converting enzyme (ACE) inhibition prevents renal interstitial inflammation and fibrosis in adult rats with unilateral ureteral obstruction (UUO) (32). This effect includes a reduction in both macrophage infiltration and collagen accumulation (30).

We wished to determine whether the timing of initiation of ACE inhibition alters the progression of renal injury resulting from UUO in the neonatal rat. We hypothesized that early inhibition of ANG II in the developing kidney with UUO would have deleterious effects, since nephrogenesis continues throughout the first postnatal week in the rat. Moreover, angiotensin AT2 receptor activity exceeds that of AT1 receptors in the first 10 days of life, and AT2 receptor stimulation is antifibrotic (1, 44). We also predicted that inhibition of angiotensin during the following 10 days would have salutary effects, since by that time nephrogenesis is complete, while AT1 receptors predominate (1) mediating pathways leading to progressive renal damage (28). A beneficial effect in adult rats with UUO has been demonstrated even when the administration of ACE inhibitor is delayed (27).

Because most clinical cases of ureteral obstruction are partial rather than complete, we studied the response to partial UUO with the use of a newly developed model (53). Chronic administration of enalapril was used to inhibit ACE either in the early (following 10 days) or late (next 10 days) neonatal period. Tubular and interstitial apoptosis was measured by the TUNEL technique, while progression of obstructive nephropathy was measured on the basis of the immunodistribution of macrophages, α-smooth muscle actin, and interstitial collagen.

MATERIALS AND METHODS

Twelve litters of Sprague-Dawley rats were subjected to partial UUO or sham operation within 48 h of birth. Surgery was performed under...
isoflurane plus oxygen anesthesia, using sterile technique, under a University of Virginia Approved Animal Protocol adhering to the NIH Guide for the Care and Use of Laboratory Animals. Partial UUO was produced by placing a 0.20-mm steel wire segment next to the left ureter at the ureteropelvic junction and tying a single 8-0 nylon suture around both ureter and the wire; the wire was then removed (53). Sham-operated animals were subjected to the same anesthesia, incision and closure. Total surgery time was 10–15 min per animal. Pups recovered on a warm surface and were returned to their mothers. Litters were divided into two groups (group I and group II, Fig. 1). All pups were treated once daily via gavage by means of a 24-gauge × 2-cm catheter (needle discarded) for younger animals, or a 22-gauge × 2.5-cm catheter for older animals. Pups in group I received either saline or enalapril (Sigma, St. Louis, MO), 30 mg/kg body wt, on days 1–10 of life and were harvested on day 21. Pups in group II received the same doses on days 11–20 and were also harvested on day 21.

On day 21, each animal was weighed and anesthetized with intraperitoneal sodium pentobarbital. The ureter and renal pelvis were measured, after which kidneys were placed in ice-cold saline, decapsulated, blotted dry and weighed before being placed in 10% buffered formalin fixative for 24 h. Kidneys were subsequently dehydrated through graded alcohols and xylene, embedded in paraffin, and sectioned at 4 μm for immunohistochemical study. Apoptosis was detected with the TUNEL technique (Apoptag in situ Apoptosis Detection Kit, Chemicon International, Temecula, CA) and DAB. Collagen was detected by picrosirius red staining as previously described (9). Mitosis was detected by the TUNEL technique as previously described (12, 13).

Morphometric measurements. Morphometric analysis for apoptosis was performed by light microscopy at a total magnification of ×400, examining 10 fields from each kidney. Apoptotic nuclei were counted and subcategorized into tubular and interstitial compartments. Macrophages, α-SMA and Sirius red staining were quantified with a computerized image-analysis program (ImagePro Plus 4.5.1, Media Cybernetics, Silver Spring, MD) and expressed as total area percentage.

Kidney homogenates and Western blots. Additional studies were performed on six 14-day-old and six 28-day-old rats subjected to sham operation, partial, or complete UUO within the first 2 days of life. Homogenates were prepared on ice with a Polytron homogenizer, using 150–200 mg frozen kidney tissue and homogenization buffer [20 mM Tris·HCl (pH 7.4), containing 0.25 M sucrose and 5 mM EDTA] including all protease and phosphatase inhibitors previously described for cell lysis buffer (31). Homogenate samples were normalized by protein concentration (100 μg protein/lane) and solubilized with boiling Laemml buffer. Proteins were separated on 10% acrylamide gels by SDS-PAGE, transferred to nitrocellulose, and prepared for immunoblot analysis as previously described (31). Equivalent protein loading per lane was verified by Ponceau stain (31). Immunoblots were incubated overnight with primary antibodies diluted 1:1,000 in 20 mM Tris·HCl, pH 7.4, 0.5 M NaCl, 1% wt/vol BSA, and 0.02% wt/vol sodium azide at 4°C on a rocking platform: mouse anti-B2 Bradykinin receptor monoclonal antibody (clone 20) from BD Transduction Laboratories; mouse anti-kallikrein KLK-1 monoclonal antibody (clone V4D11) (3) were a gift from Dr. J. Chao (USC, Charleston, SC). Immunoreactive bands were visualized with enhanced chemiluminescence (ECL) reagents according to the manufacturer’s recommendations and film exposure times ranged from 20 s to 3 min depending on primary antibody.

Statistical analysis. Data are presented as means ± SE. Comparisons between groups were made with one-way ANOVA followed by the Student-Newman-Kuels or Dunn’s test. Comparisons between left and right kidneys were made using the Student’s t-test for paired data. Comparisons between enalapril and vehicle treatment for each group were made using the Student’s unpaired t-test or the Mann-Whitney U-test. Statistical significance was defined as P < 0.05.

**Table 1. Characteristics of rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>Early</th>
<th>Late</th>
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<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Partial UUO</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>53.2 ± 1.9</td>
<td>49.2 ± 1.9</td>
</tr>
<tr>
<td>Enalapril</td>
<td>42.7 ± 3.1*</td>
<td>46.3 ± 3.9</td>
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<tr>
<td>Left kidney weight, mg</td>
<td>248 ± 13</td>
<td>229 ± 15</td>
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<tr>
<td>Enalapril</td>
<td>256 ± 28</td>
<td>288 ± 35</td>
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<tr>
<td>Right kidney weight, mg</td>
<td>267 ± 13</td>
<td>260 ± 14</td>
</tr>
<tr>
<td>Enalapril</td>
<td>269 ± 29</td>
<td>336 ± 32</td>
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Values are means ± SE. *P < 0.05, enalapril vs. vehicle.

Fig. 1. Experimental design. In the rat, nephrogenesis begins before birth but continues throughout the first postnatal week (dashed line). Animals were subjected to sham operation or partial unilateral ureteral obstruction (UUO) within the first 48 h of life. In group I, enalapril (30 mg/kg body wt) or saline vehicle was administered daily for the following 10 days of life. In group II, enalapril or vehicle was administered for 10 days beginning 10 days after surgery. Kidneys from both groups were harvested 20 days after surgery.
RESULTS

Of the 39 neonatal rats in the early treatment group treated with vehicle, 69% survived, while of the 37 treated with enalapril, 51% survived. Of the 22 neonatal rats in the late treatment group treated with vehicle, 91% survived, while of the 24 treated with enalapril, 83% survived. Treatment with enalapril increased mortality regardless of timing (P < 0.05).

As shown in Table 1, the administration of enalapril tended to decrease body weight, with differences achieving statistical significance for sham-operated rats with early treatment. There was no significant effect of enalapril treatment on kidney weight in any of the groups. As shown in Fig. 2, there was no effect of enalapril on renal pelvic dilatation regardless of timing of administration. Renal tubular and interstitial apoptosis was not significantly affected by partial UUO or the administration of enalapril (Figs. 3, A and B, and 4).

As shown in Figs. 3, C and E, and 5, macrophage infiltration increased in vehicle-treated rats following partial UUO. While early enalapril treatment did not significantly affect renal macrophage infiltration, late enalapril treatment increased mac-

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**Fig. 2.** Renal pelvic diameter. A: group I (administration of vehicle or enalapril first 10 days of life). B: group II (administration of vehicle or enalapril second 10 days of life). Sham, sham operation; partial UUO, partial unilateral ureteral obstruction; Black bars, vehicle administration; open bars, enalapril administration.

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rophage infiltration in rats with UUO (Figs. 3, C-F, and 5). As shown in Figs. 3G and 6, renal α-smooth muscle immunoreactivity increased as a result of UUO. Enalapril treatment increased the fractional contribution of α-smooth muscle staining in sham-operated rats regardless of timing of administration, while late enalapril treatment significantly increased α-smooth muscle actin staining following UUO. There was no effect of enalapril on macrophage infiltration or α-smooth muscle actin staining in the kidney contralateral to UUO (Figs. 5 and 6).

As shown in Figs. 3, K-N, and 7, partial UUO increased the deposition of interstitial collagen. Late treatment with enalapril increased interstitial collagen deposition in both kidneys following partial UUO. There was no significant effect of enalapril on collagen deposition in sham-operated animals.

As shown in Fig. 8, partial UUO resulted in there being a greater proportion of less mature glomeruli than was the case in sham-operated littermates ($P < 0.05$). Enalapril tended to further increase the proportion of intermediate glomeruli, but the differences were not statistically significant.

As shown in Fig. 9, although both apoptotic nuclei and mitotic figures are distributed both in tubule walls and interstitium (brown-staining nuclei in A and B), there is no correlation with the macrophage populations (C), which are scattered in the interstitium. Smooth-muscle actin (D) is sparse in the medulla and more generally distributed in cortex; no substantial accumulations of collagen are seen (E) that correspond either to macrophage or myofibroblast location, although thickened tubule basement membranes indicate atrophic tubules in the cortex (arrows).

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**Fig. 3.—** Continued.
To determine whether partial or complete UUO affects the expression of kallikrein-kinin system components, we compared immunoblots of kidney homogenates probed with antibodies directed against the B2 bradykinin receptor, bradykinin-directed kininogen and tissue kallikrein KLK-1. Results shown in Fig. 10 indicate temporal expression of the B2 bradykinin receptor is relatively high in the 14-day neonatal kidney, but declines by day 28. Surgical obstruction does not affect B2 receptor expression levels at day 14. However, complete UUO appears to induce expression relative to sham control and PUUO kidneys at day 28. There was no evident change in the expression of kininogen for any variable tested. Renal expression of kallikrein KLK-1 increases in day 28 kidneys relative to day 14 kidneys (Fig. 10).

**DISCUSSION**

The results of this study do not support our original hypothesis, in that early ACE inhibition with enalapril did not aggravate renal injury resulting from UUO. However, early enalapril treatment did induce macrophage infiltration and α-smooth muscle actin accumulation in kidneys of sham-operated animals. Moreover, rather than attenuating the effects of partial UUO, late enalapril treatment augmented macrophage infiltration, α-smooth muscle actin expression, and interstitial collagen accumulation. These findings provide new insight into the interaction between the response to renal injury and the development of the intra-renal RAS.
A number of studies have demonstrated the importance of an intact RAS on normal renal development. Targeted deletion of angiotensinogen, renin, ACE, or angiotensin receptors results in a wide range of renal developmental abnormalities (45). Pharmacological inhibition of the RAS also significantly impairs renal development, as revealed by the now well-established renal damage in human fetuses exposed to ACE inhibitors in utero (48, 52). The human neonate is exquisitely sensitive to angiotensin inhibition, to the degree that ACE inhibitors cause hypotension and reduction in glomerular filtration rate at concentrations ten-fold lower than those used in adults (18, 46). Administration of enalapril to rats during the first 10 days of life disrupts normal tubular and vascular growth (39), and administration of perindopril to rats during the first 14 days of life markedly reduces blood pressure and glomerular filtration rate; however, these parameters return to normal after 2 wk if the inhibitor is discontinued (5). Guron et al. (23) reported that enalapril treatment on postnatal days 3 to 13 in the rat leads to long-term impairment of urinary concentrating capacity and renal papillary atrophy, whereas treatment initiated beyond 13 days of age does not. The long-term consequences of neonatal enalapril administration include permanent renal papillary atrophy, pelvic dilatation, and alterations in renal sodium and potassium handling (20, 24). Our results in sham-operated animals are therefore consistent with these studies, as renal pathologic effects of enalapril were more marked in the early than the late treatment group. The mechanisms whereby enalapril induces renal injury in the developing kidney appear to relate at least in part to disruption of critical developmental pathways regulated by endogenous angiotensin.

We attempted to correlate a variety of changes in the renal cortical and medullary cellular responses to partial UUO in the group receiving enalapril from 10 to 20 days of age. The distribution of apoptotic and proliferating cells did not overlap that of macrophages or myofibroblasts (Fig. 9). These results are not consistent with stimulation of interstitial cell proliferation by enalapril: increased infiltration by macrophages is more likely. The increased accumulation of myofibroblasts is more likely the result of transformation of fibroblasts by an altered cytokine milieu. Moreover, the experimental design precludes the inclusion of earlier time points at which cellular proliferation may be more prominent.

In the present study, administration of enalapril from 10 to 20 days of age was injurious to the partially obstructed kidney, but earlier administration had no effect on the renal cellular response to partial UUO. Of note, however, enalapril reduces hydronephrosis and proteinuria following 21 days of partial UUO in the weanling rat (beginning ∼21–28 days of age) (2). Treatment of normal weanling rats with losartan (angiotensin AT1 receptor inhibitor) also does not result in histologic abnormalities (54). In the rat, immature stage IV glomeruli appear through postnatal day 9 (34, 35). In humans, fewer than 10% of the glomeruli are in the least mature stages by 36-wk
gestation, and by 2–5 mo of age, 50% of glomeruli are in stages IV–V, while by 6 mo over 90% are stage V (37). This suggests that timing of ACE inhibition is critical in determining the response of the developing obstructed kidney, and that renal injury appears to worsen under conditions of either too little or too much endogenous angiotensin (6). We reported previously that renal maturation is delayed by chronic complete UUO. This includes persistence of an immature pattern of glomerular maturation (7, 8), microvascular renin distribution (7), and of tubular (25) and interstitial markers (11, 38). In the present study, we showed that glomerular maturation is also delayed in the neonatal rat following partial UUO. If glomerular maturation is used as a proxy for development of the entire nephron, it would appear that the delay in renal maturation resulting from partial UUO shifts the “window” of susceptibility to angiotensin inhibition from the first 10 postnatal days to the following 10 days, as found in the present study.

Angiotensin inhibition has a salutary effect in reducing renal injury and improving renal function in adult animals with complete UUO (32). Moreover, the administration of ACE inhibitors during or after relief of UUO can also enhance recovery (19, 33, 40). Some strategies involve the combination of angiotensin blockade in concert with inhibition of parallel pathways, such as tumor necrosis factor (21), or stimulation of protective pathways, such as hepatocyte growth factor or bone morphogenetic protein (43, 56).

Because ACE (kininase II) inactivates kallidin and bradykinin in addition to converting ANG I to ANG II, we examined the effects of neonatal partial UUO on the renal expression of key components of the kallikrein-kinin system. As reported previously (15), we found that bradykinin B2 receptor abundance in the sham-operated kidney is high after birth and decreases progressively thereafter. We did not detect a maturational change in kininogen or kallikrein from 14 to 28 days of age. We found that while neonatal complete UUO increases B2 receptor abundance at 28 days, partial UUO has minimal effect on renal B2 receptor, kininogen, or kallikrein. Adult B2 knock out mice undergoing complete UUO have increased renal interstitial fibrosis, while complete UUO in transgenic rats with increased bradykinin reduces interstitial fibrosis (50). Following 5 wk of complete UUO in the adult rat, ACE activity is increased in both obstructed and contralateral kidneys, renal tissue kallikrein mRNA is markedly suppressed, and kininogens are increased (14, 16). These findings suggest that ACE inhibition may act in part through enhancing the role of the kallikrein-kinin system following complete UUO, but not following neonatal partial UUO. However, ACE inhibition does not have a differential effect on renal fibrosis in adult B2 knock out mice subjected to complete UUO (49). Taken together, the present study and other reports do not provide compelling evidence for a significant mediation of obstructive nephropathy by ACE inhibition through regulation of the kallikrein-kinin system. Others have shown an effect of enalapril to stimulate nitric oxide production through increased expression of the endothelin receptor in adult rats with short-term complete UUO (42). It is possible that in our model, enalapril mediates its effects, in part, through these additional pathways.
The present study addresses the effects of timing of ACE inhibition on the renal cellular response to partial UUO in the early postnatal period. The response to ANG II depends on the abundance and distribution of a variety of receptors, the best studied being the AT1 and AT2 receptors. Future studies employing specific inhibitors of these receptors will be necessary to distinguish their individual effects.

While the present study did not show a significant effect of enalapril on macrophage accumulation or \( \alpha \)-smooth muscle actin staining in the kidney contralateral to partial UUO, enalapril administration to rats in group II increased collagen accumulation in the contralateral kidney. It is now well recognized that the kidney contralateral to UUO behaves differently from that in a sham-operated animal. In addition to compensatory renal growth, the activity of the renin-angiotensin system is altered in the contralateral kidney, as is the activity of other pathways mediating renal interstitial fibrosis, such as bone morphogenetic protein-7 (17, 36, 57). The potential for

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**Fig. 9.** Serial consecutive sections of obstructed kidney from group II (late) enalapril-treated animal. *Left:* micrographs taken from the medulla. *Right:* micrographs taken from the outer cortex. Listed as (stain/purpose), these are: A: TUNEL/apoptosis; B: Phospho-histone/mitosis; C: ED-1/macro- phages; D: \( \alpha \)-SMA/myofibroblasts; E: picrosirius red/collagen and basement membranes. Arrows indicate atrophied tubules. *Inset:* detail of one of these tubules, which is characterized by its small diameter and thickened tubular basement membrane (indicated by arrowhead). The bars in the lowermost micrographs are applicable to all pictures in their respective column: left column = 250 \( \mu \)m, right column = 100 \( \mu \)m. Bar in E, inset = 10 \( \mu \)m.
increased vulnerability of the normal contralateral kidney to injury from ACE inhibition raises additional concerns in the use of these agents in the early postnatal period.

What are the implications of the present study for the management of human infants with obstructive nephropathy? In the rat, nephrogenesis is incomplete at birth and continues into the early postnatal period. This contrasts with human nephrogenesis, which is complete by the 34th week. The “window of vulnerability” of the maturing human kidney to injury from inhibition of angiotensin may therefore extend through the first several months of life. However, very low birth weight infants may be born with extremely immature kidneys that are still undergoing nephrogenesis. In such infants, maturation of renal function may take years rather than months (55), thereby prolonging the period of vulnerability to angiotensin inhibition. Interestingly, although difficult to extrapolate directly to this animal study, ACE inhibition has been shown to worsen the excretory phase of diuretic renography in children 2–72 mo old undergoing pyeloplasty for unilateral ureteropelvic junction obstruction (59). In a histological study of children with ureteropelvic junction obstruction, nephrogenesis was noted primarily in children 2–72 mo old undergoing pyeloplasty for unilateral ureteropelvic junction obstruction (59). In a histological study of children with ureteropelvic junction obstruction, nephrogenesis was noted primarily in children 2–72 mo old undergoing pyeloplasty for unilateral ureteropelvic junction obstruction (59). In a histological study of children with ureteropelvic junction obstruction, nephrogenesis was noted primarily in children 2–72 mo old undergoing pyeloplasty for unilateral ureteropelvic junction obstruction (59). In a histological study of children with ureteropelvic junction obstruction, nephrogenesis was noted primarily in children 2–72 mo old undergoing pyeloplasty for unilateral ureteropelvic junction obstruction (59).

GRANTS

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