

Angiotensin AT₁-receptor inhibition exacerbates renal injury resulting from partial unilateral ureteral obstruction in the neonatal rat

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Coleman CM, Minor JJ, Burt LE, Thornhill BA, Forbes MS, Chevalier RL. Angiotensin AT₁-receptor inhibition exacerbates renal injury resulting from partial unilateral ureteral obstruction in the neonatal rat. *Am J Physiol Renal Physiol* 293: F262–F268, 2007. First published April 18, 2007; doi:10.1152/ajprenal.00071.2007.—The renin-angiotensin system is activated in the developing kidney and is necessary for normal renal development, but is further activated by unilateral ureteral obstruction (UO). During nephrogenesis, there is a switch from a preponderance of angiotensin AT₂ to AT₁ receptors in the rat. We examined the renal cellular response to angiotensin II receptor inhibition in the neonatal rat subjected to partial UO under anesthesia within 48 h of birth. Group I (“early”) received saline vehicle, losartan (AT₁ inhibitor), or PD-123319 (AT₂ inhibitor) during the completion of nephrogenesis in the first 10 days of life. Group II (“late”) received each of the three treatments throughout the subsequent 10 days of life. Kidneys were harvested at 21 days, and the distribution of renin, apoptosis, macrophages, α -smooth muscle actin, and collagen was determined. Losartan and PD-123319 each increased vascular renin distribution in both kidneys. Partial UO reduced growth and increased apoptosis, macrophages, α -smooth muscle actin, and collagen in the obstructed kidney. Early losartan treatment further increased α -smooth muscle actin and collagen in the obstructed kidney and induced apoptosis, macrophages, and collagen in the contralateral kidney. Late losartan treatment had no effect on any of the parameters in either kidney, and PD-123319 had no effect on either kidney. We conclude that selective inhibition of AT₁ receptors during nephrogenesis (but not during subsequent renal maturation) exacerbates injury to the obstructed kidney and also injures the contralateral kidney. These results suggest that angiotensin II receptor blockers should be avoided in the developing hydronephrotic kidney.

hydronephrosis; nephrogenesis; apoptosis; α -smooth muscle actin; collagen

CONGENITAL URINARY TRACT OBSTRUCTION is a major cause of chronic renal insufficiency in infants and children (43). Despite intrauterine diagnosis and prompt evaluation and management of the neonate, the combination of fetal urinary tract maldevelopment and the consequences of urinary obstruction on the developing kidney lead to renal injury that is present even at the time of birth. Prevention of progression of renal injury in these infants is even more critical than in older patients with renal disorders.

The renin-angiotensin system (RAS) has been shown to play a significant role in the progression of virtually all renal disorders, and pharmacological inhibition of angiotensin II is a widely accepted therapy for attenuating or preventing renal deterioration. Since the RAS is activated to a greater extent

neonatically than at older ages, and urinary tract obstruction itself markedly activates the RAS, the rationale for angiotensin II inhibition in this setting would seem to be particularly strong (6). Chronic unilateral ureteral obstruction (UO) in the neonatal or adult rat increases renal production of transforming growth factor- β_1 (TGF- β_1) and renal interstitial fibrosis (9, 11, 24). In this regard, two recent studies have demonstrated a significant salutary effect of angiotensin-converting enzyme inhibition or angiotensin AT₁-receptor blockade on renal function in weanling rats with partial UO (2, 50).

The RAS plays a central role, not only in the progression of renal disorders, but also in normal renal development and maturation. Thus inhibition of the RAS during renal development results in abnormalities of the renal vasculature, glomeruli, tubules, and interstitium (19). We, therefore, recently described the effects of angiotensin-converting enzyme (ACE) inhibition in neonatal rats with chronic partial UO (4). This study demonstrated that the administration of enalapril during the first 10 days following partial neonatal UO (a time when nephrogenesis is continuing) did not alter injury to the obstructed kidney, whereas enalapril administered from 11 through 21 days actually aggravated renal injury (4). We postulated that these effects may have been due to a delay in renal maturation induced by ureteral obstruction, thereby shifting to a later age the “window of vulnerability” of the maturing kidney to angiotensin II inhibition (20).

The present study was designed to further elucidate the effects of angiotensin II inhibition in the neonatal rat with partial UO. During the first 10 days after birth in the rat, there is a progressive increase in the activity of renal angiotensin AT₁ receptors, and a decline in the activity of AT₂ receptors (1). In the ensuing 10 days of postnatal life, activity of AT₂ receptors is minimal, and AT₁ receptors are preponderant, as they are throughout adult life (1). We, therefore, administered either AT₁- or AT₂-receptor inhibitors to neonatal rats with chronic partial UO during the early (first 10 days) or late (ensuing 10 days) postnatal period, and measured the renal cellular responses. Despite the low abundance of angiotensin AT₂ receptors at this age, we determined the response to angiotensin AT₂-receptor inhibition in the “late” group because the receptor is present even in the adult kidney and is upregulated by sodium depletion (40). The results contrast with those generated by ACE inhibition in the same model (4) and raise new questions regarding the delicate balance of RAS activity in determining the outcome of congenital urinary tract obstruction (5).

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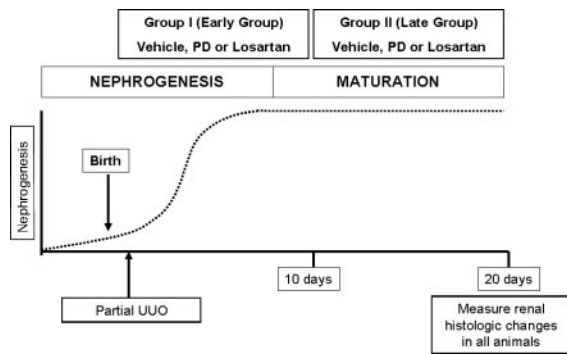


Fig. 1. Experimental design. In the rat, nephrogenesis begins before birth and continues through the first postnatal week (dashed line). Animals were subjected to partial unilateral ureteral obstruction (UUO) within the first 48 h of life. Rats in group I received either saline vehicle, PD-123319 (PD; angiotensin AT₂-receptor blocker, 10 mg/kg), or losartan (angiotensin AT₁-receptor blocker, 10 mg/kg) daily for the following 10 days of life. In group II, vehicle, PD-123319, or losartan was administered for 10 days beginning 10 days after surgery. Both kidneys from each group were harvested 20 days after surgery.

MATERIALS AND METHODS

Animals. Surgery was performed under a University of Virginia-approved animal protocol adhering to the NIH *Guide for the Care and Use of Laboratory Animals*. Ninety-five Sprague-Dawley rats (48 males and 47 females) were subjected to 0.20-mm partial UUO on day 1 following birth and returned to their mothers (4). Pups were divided into two groups (see Fig. 1). Pups in Group I received daily intraperitoneal injections of normal saline, the AT₁-receptor inhibitor losartan (Merck, Rahway, NJ), or the AT₂-receptor inhibitor PD-123319 (Sigma-Aldrich, St. Louis, MO) on days 1–10 of life and were harvested on day 21 of age. Pups in Group II were treated on days 11–20 and were also harvested on day 21 (4). Male and female animals were equally distributed among the groups. Losartan and PD-123319 were administered by intraperitoneal injection (1 μ l/g body wt) at a dose of 10 mg·kg⁻¹·day⁻¹ (32). Our laboratory has shown previously in neonatal rats with complete UUO that this dose of losartan blocks angiotensin-dependent stimulation of renal TGF- β ₁ expression, while this dose of PD-123319 blocks angiotensin-dependent stimulation of renal clusterin expression (53).

Kidney preparation. At harvest, each animal was weighed and anesthetized (intraperitoneal) with pentobarbital sodium. After the abdomen was opened, renal pelvic diameter was measured with calipers (49). Kidneys were removed to ice-cold saline and then decapsulated and blotted dry before weighing. The kidneys were then fixed in 10% phosphate-buffered formalin at RT for 24 h, after which time they were dehydrated through ethanol and embedded in paraffin. For each animal, both right- and left-side kidneys were embedded together, so that staining in both could be compared directly. Four-micrometer sections of blocks were prepared for histo- and immunohistochemical staining as detailed below.

Histochemistry. For each procedure listed below, all sections were processed together so that all were subjected to the same times of incubation and staining. The distribution of renin in afferent arterioles was established with the use of a goat polyclonal antibody (a gift from Dr. Tadashi Inagami of Vanderbilt University), applied at a dilution of 1:10,000 and treated with biotinylated secondary antibody followed by incubation with avidin-biotin complex (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA) and development with Vector VIP Substrate Kit (Vector Laboratories). The presence of apoptotic cells was determined with the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling technique (Apoptag in situ Apoptosis Detection Kit, Chemicon International, Temecula, CA), as previously described (8). Macrophages were stained with a monoclonal ED-1 antibody (CD68; Serologicals, Oxford, UK) at 1:400 dilution, with

avidin-biotin complex-diaminobenzidine development. α -Smooth-muscle actin (α -SMA) was stained with a mouse monoclonal antibody (A-2547; Sigma-Aldrich, St. Louis, MO) at a dilution of 1:800 and developed with the LSAB2 system (Dako, Carpinteria, CA) and diaminobenzidine. Collagen was detected with Picrosirius red staining, as previously described (14, 15).

Morphometry. All sections were examined in compound light microscopes equipped with QImaging cameras. Median sagittal sections of kidneys were utilized, and non-overlapping fields were examined in a pattern that sampled the entire section. Apoptotic nuclei were scored by manual counting of 10 fields at $\times 400$ total magnification, combining positive cells found in tubules and interstitium for final totals. In each group, apoptotic nuclei were approximately equally distributed between tubular and interstitial cells. Macrophage, α -SMA, and Sirius red staining were quantified with a computerized image-analysis program (Image-Pro Plus version 5.1, Media Cybernetics, Silver Spring, MD), and results are expressed as percent area.

Statistical analysis. Comparisons between groups were made by one-way analysis of variance followed by Holm-Sidak multiple-comparisons for normally distributed data and by Kruskal-Wallis one-way analysis of variance followed by Dunn's multiple comparisons for data not normally distributed. Comparisons between left and right kidneys were made using Student's *t*-test for paired data. Statistical significance was defined as $P < 0.05$.

RESULTS

As shown in Table 1, body weight was significantly reduced by losartan treatment in both groups I and II. Treatment with PD-123319 had no effect on somatic growth. Relative weight of both obstructed and contralateral kidneys was increased by early losartan treatment, but not by late treatment. Obstructed kidney weight was less than that of the contralateral kidney in the early treatment group receiving PD-123319, as was also the case in the late treatment group receiving either vehicle or losartan. Pelvic diameter of the obstructed kidney did not differ between treatment groups.

To document a known biological renal response to angiotensin II receptor inhibition, immunoreactive renin was localized in kidney sections. In the early treatment group, immunoreactive renin was restricted to the juxtaglomerular region of both kidneys in saline vehicle-treated rats (Fig. 2, A and B), but extended well down the afferent arteriole of both obstructed and contralateral kidneys following treatment with losartan (Fig. 2, E and F). Treatment with PD-123319 resulted in

Table 1. Characteristics of rats

	Saline	PD	Losartan
<i>Group I early group</i>			
BW, g	61 \pm 1	60 \pm 1	54 \pm 1*
Obstructed KW/BW ratio	5.9 \pm 0.5	5.6 \pm 0.3†	8.0 \pm 0.6*
Contralateral KW/BW ratio	6.1 \pm 0.2	6.7 \pm 0.3	7.9 \pm 0.3*
UUO pelvic diameter, mm	5.0 \pm 0.7	5.2 \pm 0.7	4.8 \pm 0.4
N	14	16	13
<i>Group II late group</i>			
BW, g	54 \pm 2	55 \pm 2	49 \pm 2*
Obstructed KW/BW ratio	6.0 \pm 0.4†	6.2 \pm 0.3	5.8 \pm 0.1†
Contralateral KW/BW ratio	6.8 \pm 0.4	6.2 \pm 0.2	6.5 \pm 0.1
UUO pelvic diameter, mm	4.3 \pm 0.5	4.8 \pm 0.6	4.2 \pm 0.5
N	16	17	19

Values are means \pm SE; N, no. of animals. PD, PD-123319, BW, body weight; KW, kidney weight; UUO, unilateral ureteral obstruction. * $P < 0.05$ vs. saline; † $P < 0.05$ vs. contralateral kidney.

patchy localization of renin along the afferent arteriole, in a pattern intermediate between vehicle and losartan treatment (Fig. 2, *C* and *D*). Renin distribution in the late treatment group was limited to the juxtaglomerular region in both kidneys of the saline-injected and PD-123319-treated animals (Fig. 2, *G–J*). Losartan treatment, however, elicited extensive renin staining in both kidneys in a pattern similar to that following early treatment (Fig. 2, *K* and *L*).

As shown in Figs. 3 and 4, 20 days of partial UUO in the neonatal rat increased renal apoptosis, macrophage infiltration, α -SMA distribution, and interstitial collagen accumulation. Early treatment of rats with losartan furthermore induced apoptosis in the contralateral kidney, but did not have a significant effect on the obstructed kidney (Figs. 3*D* and 4*A*). Early treatment with losartan also induced macrophage infiltration in the contralateral kidney, but did not have a significant effect on the obstructed kidney (Figs. 3*H* and 4*C*). Early treatment with losartan further increased the accumulation of α -SMA-containing cells in the obstructed kidney and increased the accumulation of these cells in the contralateral kidney (compared with rats treated with PD-123319) (Figs. 3, *J* and *L*, and 4*E*). Early treatment with losartan also increased the distribution of collagen in the obstructed and contralateral kidneys (Figs. 3, *N* and *P*, and 4*G*). Late treatment of rats with losartan had no effect on any of these parameters in either kidney (Fig. 4, *B*, *D*, *F*, and *H*). Treatment with PD-123319 had no effect on either kidney in both early and late groups (Fig. 4).

DISCUSSION

The major findings of the present study are that selective inhibition of angiotensin AT₁ receptors during postnatal nephrogenesis exacerbates injury to the kidney subjected to chronic partial UUO, as well as injuring the contralateral

unobstructed kidney. In contrast to ACE inhibition (4), AT₁-receptor inhibition with losartan during the subsequent period of renal maturation had no effect on the renal cellular response to partial UUO. Selective angiotensin AT₂-receptor inhibition with PD-123319 had no effect on the renal response to partial UUO, regardless of the timing of administration. The increase in vascular renin distribution following administration of either AT₁ or AT₂ blockers has been reported previously in the rat (46), and exposure of the human fetus to losartan results in a similar pattern (12). Altered distribution of immunoreactive renin confirms a biological response to the administration of losartan and PD-123319 in the present study.

Partial UUO stimulates apoptosis of the obstructed neonatal rat kidney in a degree proportional to the severity of obstruction (49). Administration of losartan during nephrogenesis increased renal apoptosis in the contralateral kidney, but did not further increase apoptosis in the obstructed kidney (Fig. 4*A*). In contrast, there was no effect of enalapril on apoptosis, regardless of the timing of administration (4). Stimulation of apoptosis by losartan could be due to unopposed stimulation of AT₂ receptors, which have been shown to mediate renal tubular apoptosis (3). In adult rats subjected to complete UUO, AT₂-receptor inhibition inhibits tubular apoptosis (36). Our laboratory has demonstrated previously that exogenous angiotensin II further increases renal apoptosis following 3 days of complete UUO in the neonatal rat, an effect suppressed by AT₂-receptor inhibition, but not AT₁-receptor inhibition (8). Our laboratory also showed that angiotensin II stimulates clusterin expression via AT₂ receptors in the neonatal rat following 3 days of complete UUO (53). Clusterin is a glycoprotein whose renal expression is increased following urinary tract obstruction, with both a proapoptotic nuclear form and a prosurvival secretory form (44). Preliminary studies show that

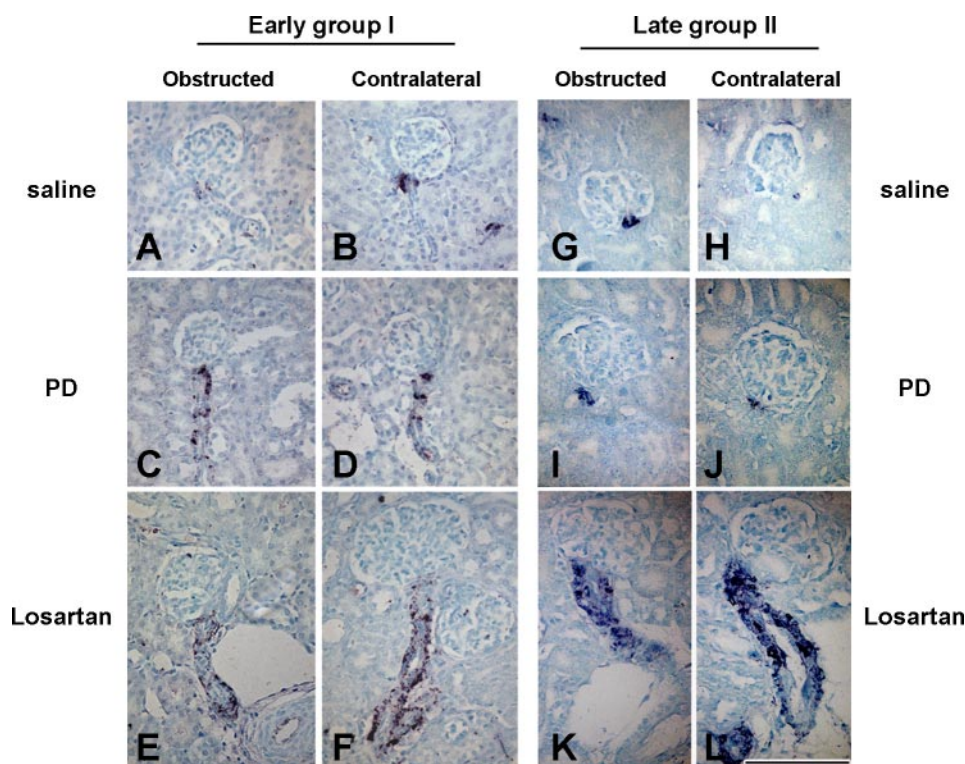


Fig. 2. Photomicrographs of immunoreactive renin distribution in representative glomeruli attached to afferent arterioles from left (*A*, *C*, *E*, *G*, *I*, *K*) and right kidneys (*B*, *D*, *F*, *H*, *J*, *L*) of each treatment for rats in groups I (*A–F*) and II (*G–L*). Immunoreactive renin appears as dark purple staining in sections counterstained with methylene blue. Scale bar in *L* represents 100 μ m.

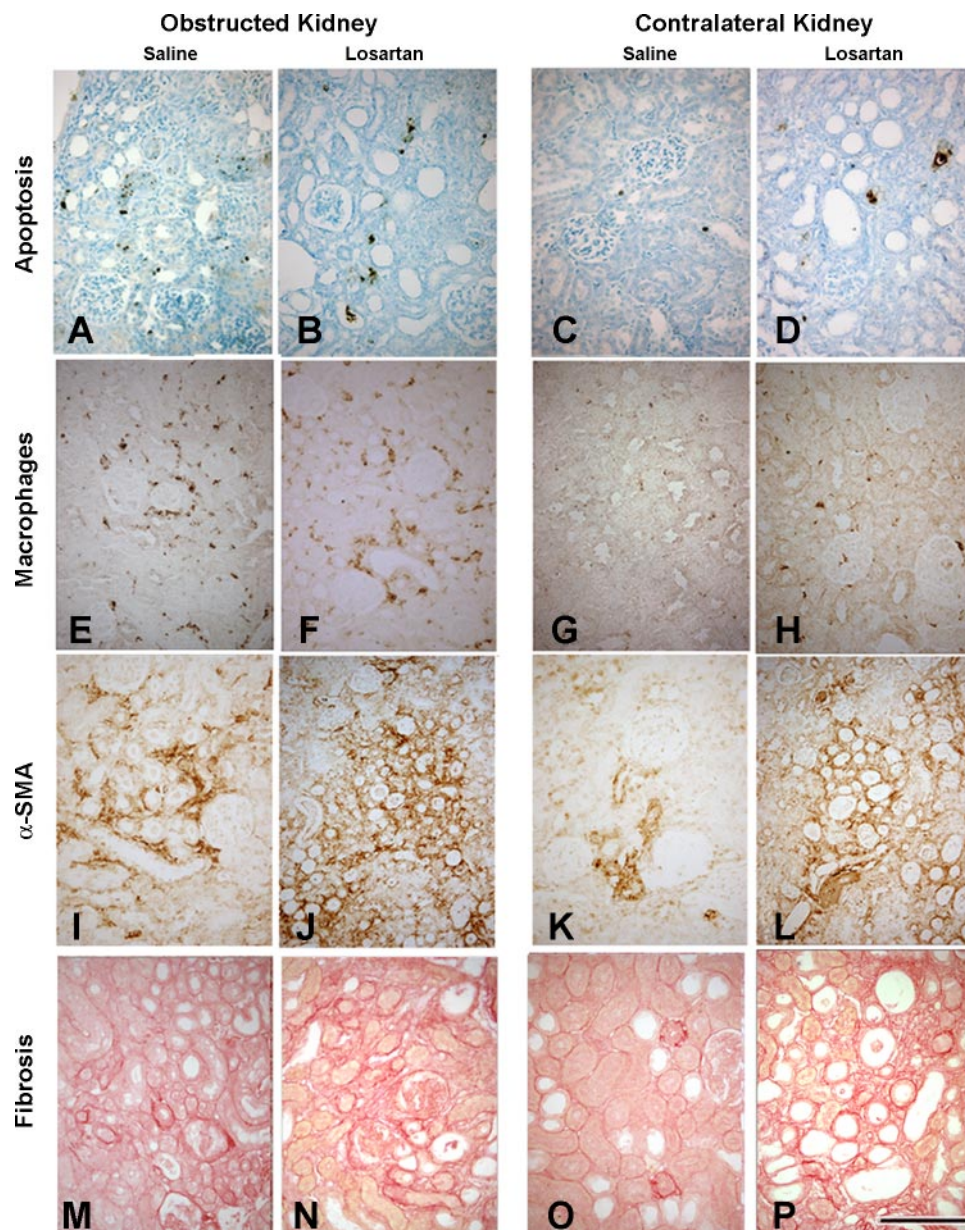


Fig. 3. Photomicrographs of representative kidney sections from obstructed (*left*) and contralateral (*right*) kidneys from saline-treated and losartan-treated rats in group I (early treatment). Animals treated with PD-123319 are not shown, since the results obtained did not differ from those of saline-treated groups. *A–D*: apoptotic nuclei are identified by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique. *E–H*: macrophages are identified by monoclonal ED-1 antibody; note the increased macrophage presence in the losartan-treated contralateral kidney. *I–L*: α -smooth muscle actin (α -SMA) is identified by a mouse monoclonal antibody. *M–P*: distribution of collagen is revealed by Picrosirius red staining. Scale bar in *P* represents 100 μ m and applies to all panels.

clusterin induces apoptosis in tubular cells by inhibiting NF- κ B-dependent Bcl-x_L production (48). The modulation of renal apoptosis by angiotensin II is complex and is dependent on the stage of renal maturation and the timing and severity of renal injury due to urinary obstruction.

Our laboratory has demonstrated previously that tubular apoptosis resulting from chronic complete UUO in the neonatal rat is mediated at least in part by macrophages infiltrating the renal interstitium (26, 27). As for apoptosis, in the present study, early losartan treatment increased macrophage infiltration of the contralateral kidney, but did not further increase macrophage density in the obstructed kidney (Fig. 4C), and there was no effect of late losartan treatment on either kidney (Fig. 4D). This contrasts with the response to ACE inhibition in this model of partial UUO in the neonatal rat, in which the density of macrophages increased markedly in the obstructed kidney following late administration of enalapril (4). Chronic

infusion of angiotensin II in adult rats activates renal monocyte chemotactic protein-1 and TGF- β ₁, leading to interstitial macrophage infiltration (39). Ishidoya et al. (22) reported that ACE inhibition suppresses macrophage infiltration in the adult rat kidney following complete UUO, but angiotensin II receptor blockade does not. While the effect of ACE inhibition was felt to be due in part to inhibition of NF- κ B (25, 35), a similar response has been described with AT₁-receptor inhibition (37) or inactivation (42). However, another report concludes that AT₂ inhibition, but not AT₁-receptor inhibition, diminishes monocyte infiltration in the obstructed kidney (16). These contrasting findings underscore the complexity of the mechanisms regulating the inflammatory response to UUO, complicated further by the changing response to angiotensin II during renal development. The presence of macrophages is not necessarily correlated with worsening renal injury: in mice lacking a functional AT₁ receptor, complete UUO results in more

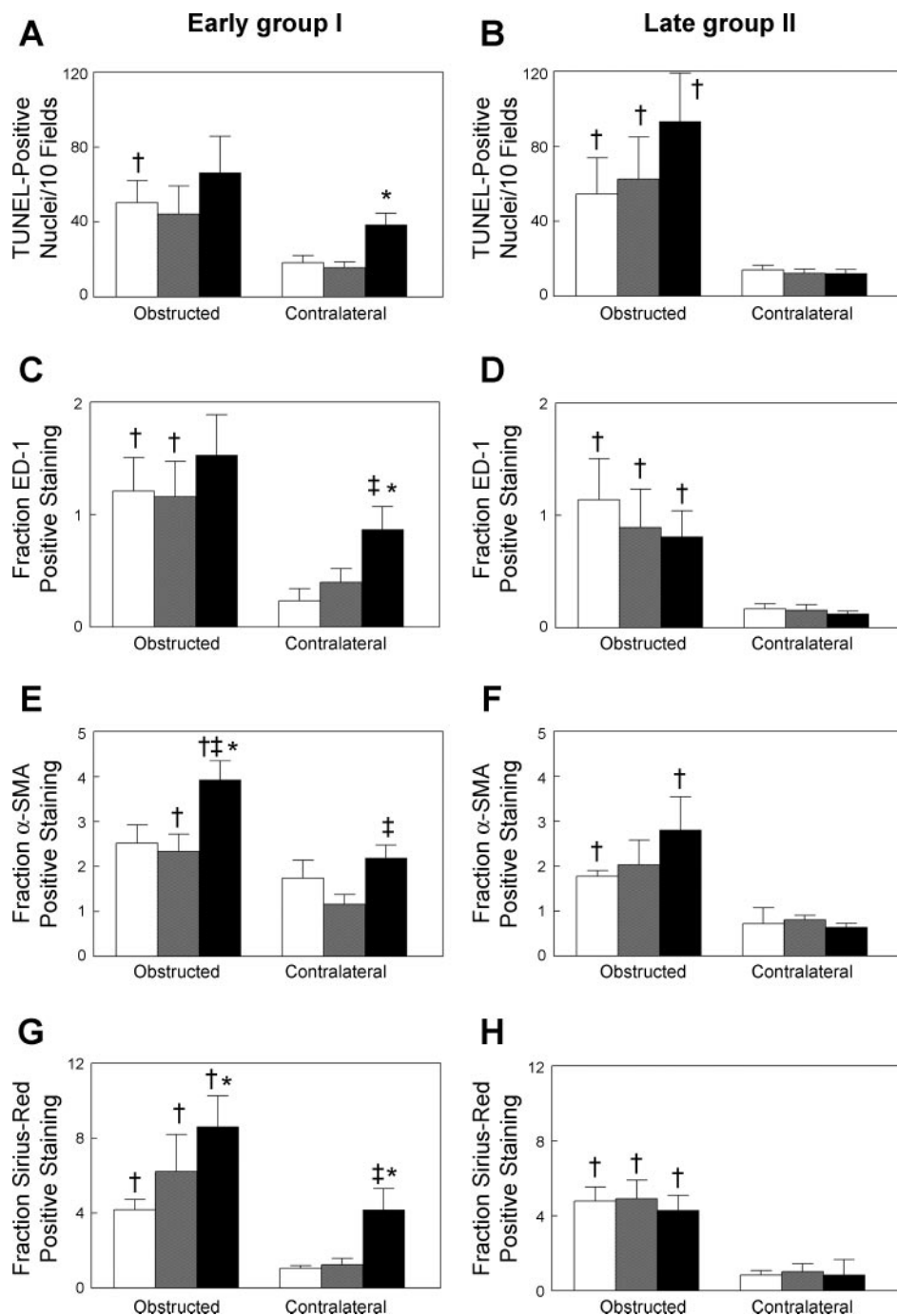


Fig. 4. Relative distribution of renal histological changes in obstructed and contralateral kidneys in early and late treatment groups (means \pm SE). Open bars represent vehicle (saline) treatment; shaded bars represent PD-123319 (AT_2 -receptor blocker) treatment; and solid bars represent losartan (AT_1 -receptor blocker) treatment. *A* and *B*: apoptotic nuclei identified by TUNEL technique. Early losartan treatment increases apoptosis in the contralateral kidney, but late treatment does not. *C* and *D*: macrophages identified by ED-1 immunostaining. Early losartan treatment results in a significant increase over the two other groups in the contralateral kidney, but this effect is absent in the late-treatment group. *E* and *F*: α -SMA identified by immunostaining. Early losartan treatment increases α -SMA immunostaining in the obstructed kidney, but late treatment does not. *G* and *H*: collagen distribution identified by Picrosirius red staining. Early losartan treatment significantly increases collagen deposition in the interstitium of both obstructed and contralateral kidneys. * $P < 0.05$ vs. vehicle treatment; † $P < 0.05$ vs. contralateral kidney; ‡ $P < 0.05$ vs. PD-123319 treatment.

severe interstitial fibrosis, despite a marked reduction in macrophage infiltration (38). Moreover, treatment of wild-type mice with losartan reduced the phagocytic activity of macrophages to a level similar to that of the AT_1 -receptor knockout mice (38).

In the rat at birth, renal interstitial fibroblasts normally express α -SMA and vimentin, which progressively disappear with maturation through the first 21 days of life (31). Following complete UUO in the neonatal rat, α -SMA expression by interstitial fibroblasts remains elevated (11). There is a significant correlation between myofibroblast transformation (the acquisition of α -SMA expression by fibroblasts) and progres-

sive increase in the deposition of extracellular matrix in obstructive nephropathy (13, 52). Thus the enhancement of renal interstitial collagen accumulation resulting from "early" administration of losartan in the present study, or "late" administration of enalapril to neonatal rats with partial UUO (4), parallels a marked increase in myofibroblast accumulation in the obstructed kidney.

Administration of losartan for the first 12 days of life in the rat causes phenotypic transformation of cells of the thick ascending limb of Henle, which is associated with increased expression of cyclooxygenase-2 (COX-2) (28). Following 24-h complete UUO in the adult rat, renal cortical COX-2 decreases,

while medullary COX-2 increases (10). In this regard, COX-2 inhibition attenuates progressive renal injury in the adult rat subjected to complete UUO (33), and losartan decreases renal COX-2 expression and reduces fibrosis resulting from UUO (30). Similarly, candesartan reduces renal medullary COX-2 induction and ameliorates renal function following release of bilateral ureteral obstruction in the adult rat (23). Taken together, the available data suggest that, in the adult rat, UUO upregulates renal medullary COX-2, and that its inhibition by losartan has a salutary effect. However, in the neonatal rat, angiotensin AT₁-receptor inhibition increases renal medullary COX-2 and aggravates renal injury, such as that observed in the present study.

It is surprising that the relatively greater AT₂-receptor activity in the first 10 days of life in the rat (40) does not confer protection from losartan-induced myofibroblast proliferation and interstitial fibrosis of obstructed and contralateral kidneys, as found in the present study (Fig. 4, E and G). Complete UUO in the adult AT₂ knockout mouse increases the accumulation of myofibroblasts and interstitial fibrosis, and this is associated with an increase in ACE activity (29, 47). Consistent with this observation, AT₂-receptor inhibition aggravates renal interstitial collagen accumulation following complete UUO in the adult rat (36), and overexpression of the AT₂ receptor ameliorates renal injury in a mouse remnant kidney model (21). It is likely that AT₂-receptor signaling in the neonatal period differs from that in the mature kidney. Moreover, the RAS can account for only 50% of interstitial fibrosis resulting from UUO in the neonatal mouse (17).

In the present study, neither losartan nor PD-123319 altered pelvic dilatation of the obstructed kidney (Table 1). Our laboratory reported previously that renal pelvic diameter is also not altered by the administration of enalapril during the neonatal period in rats subjected to sham operation or partial UUO (4). In adult rats subjected to partial UUO, administration of losartan for 7–14 days increases hydronephrosis, but does not alter the distribution of α -SMA or collagen (18), and mutant mice lacking a functional AT₁ receptor develop progressive hydronephrosis by 3 wk of age (34). While these studies suggest that stimulation of AT₁ receptors by angiotensin II is important in modulating renal pelvic volume, we were unable to show an effect of losartan on pelvic dilatation. These discrepancies may be explained by differences in the severity of partial obstruction, differences in age (adult rats vs. neonatal rats), or differences in species (neonatal mice vs. neonatal rats).

It is important to recognize that not all actions of ACE inhibitors and angiotensin II receptor blockers are mediated by the RAS as we currently understand it. Angiotensin-generating peptides include serine protease chymase, which is not regulated by ACE; angiotensin II receptors can be transactivated by the epidermal growth factor receptor, and, in addition to its action to cleave angiotensinogen to angiotensin I, renin can mediate effects through renin-specific receptors (41). Angiotensin II inhibitors can also have effects completely independent of the RAS, such as blockade of hydrolysis of AcSDKP by ACE inhibitors, or activation of peroxisome proliferator-activated receptor- γ by angiotensin II receptor blockers (41).

In conclusion, selective inhibition of angiotensin AT₁ receptors during the completion of nephrogenesis exacerbates renal tubular and interstitial injury resulting from chronic partial

UUO in the neonatal rat. Inhibition of AT₁ receptors in the maturation period immediately following the completion of nephrogenesis, however, has no significant effects on the obstructed kidney. This contrasts with the effects of ACE inhibition, which exacerbates injury to the obstructed kidney when administered during the period of maturation, but not during the completion of nephrogenesis (4). Notably, either ACE inhibition or AT₁-receptor inhibition initiated after weaning significantly improve renal function in rats subjected to neonatal partial UUO (2, 50). The switch from an injurious to a salutary effect of angiotensin II inhibition on neonatal obstructive nephropathy most likely reflects maturational changes in cell signaling. The application of these findings to congenital urinary tract obstruction in humans is complicated by species differences in the timing of nephrogenesis relative to gestation, and by the delay in renal maturation resulting from preterm birth or ongoing urinary tract obstruction itself (11, 51). While angiotensin II inhibition may attenuate the progression of obstructive nephropathy, enhanced natriuresis may augment the sodium-wasting consequences of tubular dysfunction characteristic of the obstructed kidney (7, 45) and may predispose the child to dehydration or impaired growth. Additional studies will be required to establish the optimal indications for angiotensin II inhibition in clinical congenital urinary tract obstruction.

GRANTS

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