Angiotensin stimulates TGF-\(\beta\)1 and clusterin in the hydronephrotic neonatal rat kidney

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Angiotensin stimulates TGF-\(\beta\)1 and clusterin in the hydronephrotic neonatal rat kidney. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 278: R640–R645, 2000.—Unilateral ureteral obstruction (UUO) induces activation of the renin-angiotensin system and upregulation of transforming growth factor-\(\beta\)1 (TGF-\(\beta\)1; a cytokine modulating cellular adhesion and fibrogenesis) and clusterin (a glycoprotein produced in response to cellular injury). This study was designed to examine the regulation of renal TGF-\(\beta\)1 and clusterin by ANG II in the neonatal rat. Animals were subjected to UUO in the first 2 days of life, and renal TGF-\(\beta\)1 and clusterin mRNA were measured 3 days later. Rats were divided into treatment groups receiving saline vehicle, ANG, losartan (AT1 receptor inhibitor), or PD-123319 (AT2 receptor inhibitor). ANG stimulated renal TGF-\(\beta\)1 expression via AT1 receptors, a response similar to that in the adult. In contrast, clusterin expression was stimulated via AT2 receptors, a response differing from that in the adult, in which ANG inhibits clusterin expression via AT1 receptors. We speculate that the unique response of the neonatal hydronephrotic kidney to ANG II is due to the preponderance of AT2 receptors in the developing kidney.

AT1 receptors; AT2 receptors; losartan; PD-123319

OBSTRUCTION TO URINE FLOW in early development impairs growth and maturation of the kidney and leads to nephron loss, tubular apoptosis, and interstitial fibrosis (7, 28). Chronic unilateral ureteral obstruction (UUO) leads to profound changes in the renal expression of a number of genes associated with cell proliferation, differentiation, and survival. These include marked stimulation of transforming growth factor-\(\beta\)1 (TGF-\(\beta\)1) and clusterin (6, 10). In this study, we investigated the regulation of renal TGF-\(\beta\)1 and clusterin expression after UUO in the neonatal rat. TGF-\(\beta\)1 has been shown to upregulate clusterin expression and to induce nuclear localization of clusterin (24, 35). Clusterin binds to both type I and type II TGF-\(\beta\) receptors, but not the epidermal growth factor receptor, which is increased by neonatal UUO (30, 36).

TGF-\(\beta\)1 is a cytokine that is produced in response to tissue injury and promotes cell-cell and cell-matrix contact (22, 45). Much attention has been focused recently on its role in fibrogenesis: TGF-\(\beta\)1 stimulates cellular production of extracellular matrix components, as well as inhibiting matrix degradation (37). Thus inhibition of TGF-\(\beta\)1 reduces renal interstitial fibrosis, a major long-term consequence of chronic UUO (2). TGF-\(\beta\)1 also stimulates cellular apoptosis, another deleterious renal consequence of chronic UUO (4, 6, 44).

However, TGF-\(\beta\)1 also plays a salutary role in the cellular response to injury. By promoting extracellular matrix synthesis after ischemic injury, TGF-\(\beta\)1 provides regenerated tubular cells a substrate for adhesion, migration, and exposure to ligands that participate in the recovery process (3). In addition, TGF-\(\beta\)1 is a potent immunosuppressant: blocking TGF-\(\beta\)1 expression could aggravate the interstitial inflammatory response present as a result of UUO (17).

Clusterin, a large glycoprotein also produced in response to tissue injury, plays a role in cellular aggregation and has been associated with protection from apoptosis (15) and shown to induce aggregation of renal tubular cells in vitro (41). By preventing cellular detachment resulting from tubular injury, clusterin may reduce apoptosis activated by separation of the cell from its basement membrane, a process termed “anoikis” (16). In addition, clusterin plays a role in countering oxidant injury, which contributes to the renal damage resulting from UUO (39, 51).

Chronic UUO also results in the stimulation of the renin-angiotensin system, which is already highly activated in the fetus and neonate compared with the adult (20). In addition to its role as a vasoconstrictor, ANG II has been shown to act as a growth factor in the kidney, regulating both cellular proliferation and programmed cell death (apoptosis), depending on the distribution and subtype of receptors (43, 47). Thus stimulation of cellular proliferation is largely mediated by AT1 receptors, whereas apoptosis or inhibition of proliferation is mediated by AT2 receptors (43, 47).

We and others (11, 23, 34) have shown that TGF-\(\beta\)1 expression is regulated by ANG II, and that ANG-converting enzyme inhibitors or AT1 receptor inhibitors reduce renal TGF-\(\beta\)1 expression by the hydronephrotic kidney and decrease interstitial fibrosis. We have also shown that inhibition of AT1 receptors for 14 days by losartan stimulates renal clusterin expression in the neonatal rat, suggesting that endogenous ANG II inhibits clusterin expression through stimulation of AT1 receptors (11). The present study was designed to examine the regulation of TGF-\(\beta\)1 and clusterin gene

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expression by ANG II in the initial phase of UUO in the neonatal rat. A 3-day duration of UUO was used, as both AT1 and AT2 receptors are abundant in the kidney at this age and renal AT2 expression falls rapidly in the neonatal period (27, 48). Selective inhibitors of AT1 and AT2 receptors were used to reveal their respective role of AT1 receptors in regulation of transforming growth factor (TGF)-β1 and clusterin expression in neonatal hydronephrotic kidney. Exogenous ANG II was administered using timed-release pellets (0.5 mg·kg−1·day−1). Although we could not measure blood pressure in neonatal rats, this dose is nonhypertensive in adult rats (49) and would be expected to be nonhypertensive in the neonate as well, in view of the lower pressor response to ANG II in the neonate (40). Control rats received daily injections of saline vehicle, and rats not receiving PD-123319 or ANG II received placebo timed-release pellets. Thus each animal received an identical number of injections and pellets.

Seventy-two hours after UUO, rats were killed by lethal injection of pentobarbital sodium. Kidneys were immediately removed, decapsulated, and processed for RNA analysis as described previously (11). Briefly, RNA was extracted using guanidinium isothiocyanate (9), and 10-µg samples were subjected to Northern blot analysis and hybridized with a rat TGF-β1 cDNA (gift of Su Wen Quan, National Cancer Institute, Bethesda, MD), clusterin cDNA (gift of M. Tenniswood, University of Ottawa, Ottawa, Ontario, Canada), and a 780-bp cDNA fragment of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; American Type Culture Collection, Rockville, MD). The latter was used as a housekeeping gene to control for equal loading. Hybridization signals were determined by autoradiography and quantitated by densitometry. The ratio of TGF-β1 and clusterin mRNA to GAPDH mRNA was calculated for each kidney in each group. Statistical analysis. Data are presented as means ± SE. Comparisons between UUO and intact kidneys were made using the t-test for paired data. The effects of exogenous ANG II and of AT1 or AT2 inhibitor were determined separately for UUO and intact opposite kidneys using one- and two-way ANOVA. Statistical significance was defined as P < 0.05.

RESULTS

Mean body weight per rat pup ranged from 7 to 9 g and did not differ between groups. For all groups, renal TGF-β1 expression by the obstructed kidney exceeded that of the intact opposite kidney (see Fig. 3, A and B). This indicates that UUO induces expression of TGF-β1 by the ipsilateral kidney.

As shown in Figs. 2 and 3, A and B, and Table 1, administration of exogenous ANG further increased renal TGF-β1 expression in the obstructed kidney, but not the intact opposite kidney. As revealed by two-way ANOVA (Table 1), this additional increase was prevented by losartan but not by PD-123319. This indicates that exogenous ANG boosts the expression of TGF-β1 in the obstructed kidney by binding to the AT1 receptor. Administration of losartan minimally reduced the baseline renal expression of TGF-β1 by both the
obstructed and intact opposite kidney (Fig. 3A, Table 1), whereas infusion of PD-123319 had no significant effect (Fig. 3B, Table 1). This indicates that endogenous ANG stimulates TGF-β1 expression by binding to AT1 receptors in both obstructed and intact opposite kidneys. It should be noted that by one-way ANOVA, TGF-β1 expression was not different between ANG and PD-123319 and saline treatment groups (Fig. 3B). This suggests that TGF-β1 expression may also be stimulated by AT2 receptors.

As shown in Figs. 2 and 3, C and D, UUO alone did not affect ipsilateral renal clusterin expression, whereas ANG administration significantly increased clusterin expression by the obstructed but not the intact opposite kidney (Table 1). In contrast to its effect on TGF-β1, losartan did not decrease the ANG-induced stimulation of clusterin expression (Fig. 3C, Table 1). In contrast, PD-123319 prevented the response to exogenous ANG (Fig. 3D, Table 1). This indicates that exogenous ANG boosts the expression of clusterin in the obstructed kidney by binding to the AT2 receptor. This effect of PD-123319 was greater after administration of exogenous ANG than on baseline clusterin expression in the obstructed kidney (Fig. 3D, Table 1).

DISCUSSION

We previously reported that UUO in the neonatal rat causes a progressive increase in ipsilateral renal TGF-β1 expression throughout the first month of life (10). The results of the present study confirm the stimulation of renal TGF-β1 expression by UUO and reveal that a significant component of its expression is dependent on ANG II AT1 receptors. A novel finding is the demonstration of additional renal TGF-β1 expression by exogenous ANG II in the obstructed kidney but not the intact opposite kidney. This indicates that the upregulation of TGF-β1 by ANG II is enhanced in the obstructed kidney, an effect that occurs in the face of normalization of receptor expression after 3 days of UUO (48). One explanation for this may relate to the demonstration of enhanced TGF-β1 production by stretching cultured mesangial cells, a response that is enhanced in the presence of ANG II (21). Because TGF-β1 is also produced by tubular cells (1), which are stretched by tubular dilatation after UUO, a similar mechanism may underlie our observations. Renal tubular cells express ANG AT1 and AT2 receptors, as well as TGF-β1 receptors (1, 32, 33). In view of the lack of effect of 3 days of UUO on AT1 and AT2 receptor mRNA in either obstructed or contralateral kidneys (48), it is likely that these effects are mediated by signaling downstream from ANG binding with its receptors and modulated by the stretched tubules in the obstructed kidney.

We documented a linear correlation between the duration of UUO and renal mRNA expression of TGF-β1

Table 1. Statistical comparison between treatment groups

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<td>Clusterin</td>
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P values for comparisons by 2-way ANOVA. Unilateral ureteral obstruction (UUO) and intact kidneys compared separately. TGF, transforming growth factor; NS, not significant.
in the neonatal rat (10), and the intensity of tubular immunoreactive TGF-β1 is also increased in proportion to the duration of obstruction (8). In addition to its transcription and translation, activation of latent TGF-β1 also plays a role in its biological action. In this regard, ANG II not only stimulates the synthesis of latent TGF-β but also promotes its conversion to the biologically active form and increases the expression of type-1 TGF-β receptor (18, 19).

In light of the significant role played by TGF-β1 in the progression of interstitial fibrosis, there is considerable interest in modulating its expression through regulation of ANG II. We demonstrated that 14 days of inhibition of AT1 receptors with losartan reduced TGF-β1 expression in the obstructed neonatal rat kidney by 30%, without affecting expression in the intact kidney (11). Twenty-eight days after the relief of 5 days of UUO in the neonatal rat, renal interstitial fibrosis was markedly attenuated compared with the persistently obstructed kidney, whereas microvascular renin and tubular TGF-β1 expression decreased significantly (8). ANG II has been shown to stimulate TGF-β1 expression by renal interstitial fibroblasts through AT1 receptors and also to upregulate angiotensinogen in the fibroblasts themselves (38). We have shown that chronic administration of ANG II in the adult rat stimulates the expression of TGF-β1 in glomeruli and tubules and increases interstitial fibrosis (49).

We reported also that TGF-β1 expression in the obstructed kidney of the neonatal mouse increased in animals with one to four functional copies of the angiotensinogen gene, but not in those lacking a functional angiotensinogen gene (14). Of interest, whereas the degree of renal interstitial fibrosis was directly related to the number of copies of angiotensinogen (from 0 to 2 copies), the reduction in fibrosis in mice without a functional renin-angiotensin system was only 50% (14). Moreover, using in situ hybridization histochemistry, others have demonstrated increased renal cortical interstitial TGF-β1 mRNA and spontaneous interstitial fibrosis in mice lacking functional copies of the angiotensinogen gene (31). These findings suggest that factors other than ANG and TGF-β1 also contribute to the progression of interstitial fibrosis.

We have shown that clusterin mRNA increases progressively throughout the first month of life in the neonatal rat kidney subjected to UUO, whereas clusterin expression in the intact kidney remains at baseline levels (5). Although renal clusterin expression was not stimulated by 3 days of UUO, clusterin was boosted additionally by exogenous ANG II. The stimulation of renal clusterin expression by ANG is a novel finding and is surprising in light of our previous reports showing stimulation of clusterin expression by losartan or enalapril in the obstructed or intact kidney of the 14-day-old rat (11, 50). The present study shows clearly that the ANG-dependent stimulation of renal clusterin expression in 3-day-old rats was mediated via the AT2 receptors. Moreover, the response was limited to the obstructed kidney. Immunoreactive clusterin follows the same pattern, and relief of UUO at 5 days attenuates the distribution of clusterin-positive tubules at 1 mo of age (8). Thus the distribution of the glycoprotein parallels the abundance of renal clusterin mRNA.

The stimulation of clusterin by ANG in the present study can be explained by a maturational shift in the balance between renal AT1 and AT2 receptors. At 1 day of age, the relative abundance of renal mRNA for the AT2 receptor is 10-fold greater than that of the AT1 receptor, whereas the distribution is virtually equal at 7 days (27). By 14 days, however, renal AT2 receptor expression has decreased 30-fold below that of AT1 receptors (27). We have shown that renal AT1 and AT2 receptor binding correlates well with abundance of steady-state mRNA: as a result of 24 h of UUO in the neonatal rat, both AT1 and AT2 receptors are downregulated in the obstructed kidney (48). However, by 3 days of UUO, renal AT1 and AT2 receptor expression is not different from that of the intact kidney, whereas by 28 days of UUO, AT1 receptor mRNA and binding have increased compared with the intact kidney (48). Thus in the 3-day-old neonatal rat kidney, there is a preponderance of AT2 receptors, whereas in the 14-day-old animal, there is a marked preponderance of AT1 receptors.

As shown in the proposed scheme in Fig. 4, ANG II stimulates renal AT2 receptors in the obstructed kidney of the 3-day-old rat, resulting in upregulation of clusterin. PD-123319 blocks this response, whereas losartan has no effect because of the relative paucity of AT1 receptors. In the 14-day-old rat, ANG II stimulates the more abundant renal AT1 receptors, which attenuate clusterin expression. Either losartan (which reduces AT1 receptor activation) or enalapril (which reduces endogenous ANG II) increases renal clusterin expression in the 14-day-old rat by reducing the ANG-mediated inhibition (11, 50). Although the magnitude is smaller, the renin-angiotensin system is activated by UUO in the adult as well as in the neonate (6), and endogenous ANG would be expected to reduce, rather than to stimulate, clusterin expression in the obstructed kidney. Thus, although ANG II clearly modu-

![Fig. 4. Proposed scheme of effects of maturation on regulation of renal clusterin expression by ANG II. Relative abundance of ANG AT1 and AT2 receptors is denoted by size of circles. Left: relative distribution of renal ANG receptors in rats at 3 days of age; right: distribution in rats at 14 days through adulthood. Stimulation of AT1 receptors downregulates clusterin, whereas stimulation of AT2 receptors upregulates clusterin.](image)
lates renal clusterin expression, other factors are responsible for the primary initiation of clusterin upregulation after UUO.

In summary, UUO in the neonatal rat activates the intrarenal renin-angiotensin system and induces the renal expression of TGF-β1 and clusterin. TGF-β1 is upregulated through stimulation of the AT1 receptors by endogenous or exogenous ANG II. Despite the preponderance of renal AT2 over AT1 receptors in the first week of life and the shift to a preponderance of renal receptors from AT2 to AT1 by the second week, ANG continues to stimulate renal TGF-β1 expression in the 14-day-old and adult rat, as it does in the first 3 days of life (11, 49). In contrast, in the first 3 days of life, clusterin expression by the obstructed kidney is upregulated by exogenous ANG II through stimulation of the AT2 receptors but not by AT1 receptors. As reported by us previously, by the second week of life, ANG inhibits clusterin expression by the obstructed kidney, an effect mediated by the AT1 receptors (11).

Perspectives

We propose that the maturational shift in renal expression of ANG receptors from AT2 to AT1 explains the developmental change in regulation of renal clusterin expression by ANG II. The scheme proposed in Fig. 4 depicts opposing effects of ANG II binding to AT1 and AT2 receptors. This paradigm has been established for other signaling pathways for AT1 and AT2 receptors. Thus vasoconstriction, sodium retention, and cell proliferation are mediated by AT1 receptors and the opposing signaling response of AT2 receptors. This paradigm has been established for other signaling pathways for AT1 and AT2 receptors. Thus vasoconstriction, sodium retention, and cell proliferation are mediated by AT1 receptors and apoptosis are mediated by AT2 receptors (12). In view of the ubiquitous distribution of tissue renin-angiotensin systems, it is likely that the opposing signaling responses of AT1 and AT2 receptors account for a number of maturational changes in the response to injury.

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