Renal apoptosis parallels ceramide content after prolonged ureteral obstruction in the neonatal rat

RAJESH K. MALIK, BARBARA A. THORNHILL, ALICE Y. CHANG, SUSAN C. KILEY, AND ROBERT L. CHEVALIER

Department of Pediatrics, University of Virginia, Charlottesville, Virginia 22908

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Malik, Rajesh K., Barbara A. Thornhill, Alice Y. Chang, Susan C. Kiley, and Robert L. Chevalier. Renal apoptosis parallels ceramide content after prolonged ureteral obstruction in the neonatal rat. *Am J Physiol Renal Physiol* 281: F56–F61, 2001.—Obstructive nephropathy, the primary cause of renal insufficiency in infants, is characterized by progressive renal apoptosis. Ceramide is a sphingolipid known to stimulate apoptosis in the kidney. We investigated the effects of unilateral ureteral obstruction (UUO) on endogenous renal ceramide content and apoptosis in neonatal and adult rats. Animals were subjected to UUO or sham operation on the first day of life and were studied 3–28 days later. Adult rats were similarly treated and then studied 3 or 14 days later. In additional neonatal rats, the obstruction was removed after 5 days, with study at 14 or 28 days. Renal ceramide content was measured by diacylglycerol kinase assay, and apoptosis was determined by the terminal deoxynucleotidyl transferase dUTP nick-end-labeling technique. Renal ceramide content was 50-fold higher in the 3-day neonatal compared with the adult kidney and 10-fold higher in the 7-day neonatal compared with the adult kidney, but there was no additional effect of UUO on ceramide content at these ages. However, after 14 or 28 days UUO in the neonate, renal ceramide was elevated compared with sham or intact opposite kidneys, and renal apoptosis was directly related to ceramide content (r = 0.99, P < 0.001). Moreover, renal ceramide was reduced by relief of obstruction (P < 0.05). There was less apoptosis in the obstructed kidney of the adult than the neonate, and UUO had no effect on ceramide content at 14 days in the adult. We conclude that prolonged UUO (at least 14 days duration) increases endogenous renal ceramide in the neonatal but not the adult rat. It is likely that this contributes to the prolonged renal apoptotic response of the neonatal obstructed kidney.

Obstructive nephropathy; hydronephrosis; sphingolipids; renal development

Obstructive nephropathy constitutes the major cause of renal insufficiency and renal failure in the infant and child and remains a major factor contributing to renal dysfunction in the adult. There is increasing evidence that destruction of renal tubular cells by apoptosis (programmed cell death) resulting from urinary tract obstruction leads to tubular atrophy, one of the hallmarks of obstructive nephropathy (17). Moreover, the severity of the apoptotic response to unilateral ureteral obstruction (UUO) is far greater in the neonatal than the adult rat, a factor that is likely to contribute to the impaired growth of the chronically obstructed developing kidney (4).

In view of the significant role of apoptosis in the pathogenesis of the renal cellular injury resulting from urinary tract obstruction, there is great interest in factors regulating the renal apoptotic response. Relief of chronic UUO in the neonatal rat reduces apoptosis in the ipsilateral kidney by 50% (8). We have demonstrated a role for altered growth factor expression, such as a reduction in renal epidermal growth factor factor by the obstructed kidney (14, 15). Chronic administration of exogenous epidermal growth factor or insulin-like growth factor-1 reduces apoptosis and tubular atrophy in the obstructed kidney (5–7). We have also reported an inverse correlation between the tubular production of the antiapoptotic oncoprotein bcl-2 and regional apoptosis of renal tubular epithelial cells in the obstructed kidney (9).

Attention is now focusing on potentially harmful stresses affecting the kidney injured by continued urinary tract obstruction. It appears that stretching of the renal tubular cells by transmitted increased hydrostatic pressure can provide a powerful mechanical stimulus to apoptosis in the obstructed kidney (31). Ischemia is another stimulus to apoptosis, and UUO induces a profound reduction in renal blood flow and impairment of autoregulation of renal blood flow (10, 30). Moreover, reactive oxygen species are known to reduce the threshold of tissues to undergo apoptosis (25), and reactive oxygen species are significantly increased in the chronically obstructed kidney (24).

Recently, the sphingolipid ceramide has been identified as a molecule that can act as a potent stimulus to apoptosis (35). When present in high concentrations, ceramide, one of the most hydrophobic molecules in the cell, can induce apoptosis and disrupt nephrogenesis (36). Ceramide synthesis has been described in glomerular endothelial cells, mesangial cells, and tubular epithelial cells (21, 22, 40). The developing kidney is an

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Address for reprint requests and other correspondence: R. L. Chevalier, Dept. of Pediatrics, PO Box 800386, Univ. of Virginia, Health Sciences Ctr., Charlottesville, VA 22908 (E-mail: rlc2m@virginia.edu).

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organ that normally undergoes extensive remodeling in fetal and early postnatal life, a process that involves widespread apoptosis (27). We have demonstrated that the very high prevalence of renal apoptosis in the fetal and neonatal rat is associated with similarly elevated levels of intrarenal ceramide, and both ceramide production and endogenous renal apoptosis decrease to adult levels during the first month of life (28). This phenomenon is tissue specific: there are far lower levels of ceramide production in the developing lung and liver than in the kidney (28).

The present study was designed to investigate the relationship of renal apoptosis to endogenous renal ceramide production in response to chronic UUO in the rat. In view of the marked developmental differences in renal apoptosis after UUO in the neonate compared with the adult, as well as the developmentally determined changes in endogenous renal ceramide production, we examined the response of both newborn and adult animals. In addition, to examine the effects on ceramide production when apoptosis is modulated in the obstructed kidney, additional animals were subjected to sham operation or UUO and studied 3 or 14 days later. In 11 neonatal animals, the obstruction was removed after 5 days as described previously (12), and animals were killed at 14 or 28 days after relief of obstruction. Kidneys were excised, placed in ice-cold saline, then blotted dry and weighed before lipid extraction or quantitation of apoptosis. For 3- and 14-day neonates, separate bisected and half were used for lipid extraction and half for quantitation of apoptosis were removed as described above (1). Ceramide levels were determined by diacylglycerol kinase assay, as described (33, 37), and quantitated against a standard curve using a phosphorimager. Ceramide levels were normalized for lipid phosphate, corrected for weight of the organs, and are expressed as picomoles ceramide per nanomole phosphate per gram of organ weight.

Quantitation of apoptosis. The kidneys to be utilized for quantitation of apoptosis were removed as described above and fixed in 10% phosphate-buffered formalin for 24 h. The fixed kidneys were dehydrated through graded alcohol and xylene and embedded in paraffin, and 5-μm sections were obtained. Apoptotic nuclei were labeled using the terminal deoxynucleotidyl transferase dUTP nick-end-label (TUNEL) technique (Apoptag; Oncor, Gaithersburg, MD) as previously described (4). Twenty percent of the kidney area in nonoverlapping fields was scanned, and the total number of apoptotic nuclei was counted.

Statistical analysis. Data are presented as means ± SE. The relationship of apoptosis to duration of obstruction and to ceramide content was determined by linear regression analysis. Comparisons between left and right kidneys were performed by Student’s t-test or the Mann-Whitney rank sum test for paired samples. Comparisons among sham, UUO, and postobstructed kidney (and among age groups) were performed by one-way analysis of variance followed by the Student-Newman-Keuls pairwise multiple comparison test or Kruskal-Wallis one-way analysis of variance followed by Dunn’s multiple comparison test using Sigma Stat Version 2 (Jandel Scientific, San Rafael, CA). Statistical significance was defined as P < 0.05.

RESULTS
As shown in Fig. 1A, body weight increased progressively during the period of study and was unaffected by UUO. Kidney weight increased as a result of ipsilateral UUO in the neonatal rat after 3 days of obstruction but decreased after 14 or 28 days of obstruction, with the intact kidney demonstrating compensatory growth (Fig. 1B). Although body weight was greater in rats undergoing relief of UUO than in sham-operated or persistently obstructed rats, kidney weight did not
differ from those in persistently obstructed animals (Fig. 1). In contrast, kidney weight of the adult rats subjected to ipsilateral UUO for either 3 or 14 days was greater than that of sham-operated animals, and there was compensatory growth at 14 days.

As shown in Fig. 2A, the ceramide content of the 3-day-old neonatal kidney was 50-fold that of the adult kidney, and there was no significant effect of UUO on either kidney at either age. Total renal apoptosis increased 4-fold by 3 days of ipsilateral UUO in the neonate and over 10-fold in the adult, and the density of apoptosis in the neonate was double that in the adult (Fig. 2B). After 14 days UUO in the neonate, renal ceramide content in the ipsilateral kidney was double that of the sham-operated kidney and fourfold that of the intact opposite kidney (Fig. 2C). In contrast, there was no effect of UUO on total ceramide content in the adult kidney (Fig. 2C). There was no significant difference between ceramide content in the neonatal compared with adult kidney from sham-operated animals or for the intact opposite kidney 14 days after UUO (Fig. 2C). Apoptosis increased sixfold after ipsilateral UUO in the neonate and fourfold in the adult, with a significantly greater increase in the neonate (Fig. 2D). There was no significant difference between renal apoptosis in the neonatal compared with adult kidney from sham-operated animals or for the intact opposite kidney 14 days after UUO (Fig. 2D).

As shown in Fig. 3A, apoptosis increased progressively throughout the first month of life in the kidney with ipsilateral UUO since the first day of life. The rate of increase was greater in the first 2 wk than in the third and fourth weeks. In the neonatal kidney after 14 or 28 days of UUO or sham operation, there was a direct correlation between renal apoptosis and renal ceramide content (Fig. 3B).

As shown in Fig. 4, renal ceramide content decreased by >80% from 7 to 14 days in the sham-operated or intact kidney of the postnatal rat (open or hatched bars). Thus the developmental decrease in the renal ceramide content does not approach the adult levels until 14 days of age. However, compared with the sham-operated and intact opposite kidneys for each age, ipsilateral UUO induced an increase in renal ceramide content to 50 pM·nM phosphorus⁻¹·g KW⁻¹ by 14 days and to 91 nM phosphorus⁻¹·g KW⁻¹ by 28 days (filled bars), where KW is kidney weight. Compared with the persistently obstructed kidney, relief of UUO reduced the renal ceramide content by 60% either 14 or 28 days after relief of the obstruction. Thus UUO upregulates renal ceramide content, and relief of obstruction reverses this effect.

**DISCUSSION**

This study explores for the first time the effects of urinary tract obstruction on ceramide content of the
hydronephrotic kidney and its relationship to renal apoptosis. Although the relative contribution of ceramide to renal apoptosis could not be determined, exposure of metanephric kidneys to ceramide has been shown to stimulate apoptosis in tubules as well as mesenchyme (36). A novel finding in the present study is the importance of the maturational stage of the kidney on the accumulation of ceramide induced by UUO and its relationship to apoptosis. We have reported previously that renal apoptosis parallels renal ceramide content in the developing rat kidney, with ceramide falling over 100-fold from the fetus through the first postnatal week (28). This was confirmed in the present study as well, as shown for 3- and 7-day-old rats compared with adults (Figs. 2A and 4). At this early age, endogenous renal ceramide content remains 50-fold greater than that of adults, and 3–7 days of UUO have no additional effect on ceramide content. The stimulation of renal apoptosis by UUO in the first week of life is clearly independent of renal ceramide content and suggests that, at this age, highly elevated ceramide levels may play a greater role in regulating apoptosis associated with developmental tissue remodeling than in the early apoptotic response to injury resulting from UUO. The potential stimulation of ceramide accumulation by UUO in the immature hydronephrotic kidney is a late phenomenon, detectable only after 14 days of obstruction, and even greater after 28 days of obstruction (Fig. 4). The increased renal ceramide content of the obstructed neonatal kidney may also be viewed as a delay in the normal maturational decrease in renal ceramide content. In this regard, we have demonstrated previously in the neonatal rat that chronic UUO delays a number of maturational characteristics of the developing kidney. These include persistence of a fetal pattern of renin-containing afferent arteriolar cells, immature glomeruli, vimentin expression by renal tubular cells, and α-smooth muscle expression by renal interstitial cells (3).

Apoptosis in the obstructed neonatal kidney also increases progressively during the first month of UUO (Fig. 3A) and closely parallels the increase in ceramide content after 14–28 days of obstruction (Fig. 3B). We have shown that relief of 28 days of UUO in the neonatal rat decreases apoptosis by 50% compared with persistent UUO (8). This reflects the reduction in renal ceramide content resulting from relief of obstruction (Fig. 4). It should be noted that after UUO in the neonate, ~50% of the apoptotic cells are tubular epithelial cells and 50% interstitial cells (with virtually no detectable apoptotic vascular or glomerular cells) (8). In the adult rat, UUO results in an increase in apoptotic tubular epithelial cells at 14 days, with a decrease to baseline by 45 days (13). However, apoptosis of interstitial cells continues to increase through 45 days of UUO in the adult (13). It is therefore possible that tubular cells are more sensitive to ceramide-induced apoptosis than are interstitial cells.

The production of ceramide in the kidney can result either from sphingomyelin degradation or from de novo synthesis. Renal ischemia followed by reperfusion results in a transient reduction in ceramide content,
followed by maintenance of supranormal levels (40). This response appears to be due to increased ceramide synthesis rather than to sphingomyelin degradation (39). Renal tubular cells exposed to oxalate accumulate ceramide as a consequence of increased sphingomyelinase activity (2). Cellular ceramide is also increased by exposure to other oxidants, a response that could be attenuated by pretreatment with antioxidants (2). Of interest, ceramide increases the rate of transcription of the manganese superoxide dismutase (MnSOD) gene, which may serve as a protective cellular response (32). It is therefore likely that enhanced accumulation of ceramide in the obstructed kidney results, at least in part, from the generation of reactive oxygen species (24). The neonatal obstructed kidney may be particularly susceptible to the generation of reactive oxygen species, because endogenous renal antioxidant enzymes, including superoxide dismutase, are suppressed in the neonate (18) and are further suppressed by UUO (26).

Ceramide-induced apoptosis in the immature hydronephrotic kidney may be mediated by p53, a tumor suppressor gene (34). This is plausible, because oxygen free radicals cause DNA strand breaks (a major inducer of p53), and renal p53 expression is stimulated by UUO (16, 29). Ceramide has also been shown to activate the CD95 (Fas) pathway to apoptosis (19), and this pathway mediates apoptosis of the distal tubule after UUO (20). Ceramide generated by activation of sphingomyelinas has been shown to mediate hypoxic cell death (38). This effect can be inhibited by the oncoprotein bcl-2, which reduces ceramide formation and caspase activation (38). This is of particular interest, because we have reported recently that chronic UUO suppresses renal tubular bcl-2 (9).

There was no correlation between renal apoptosis and ceramide content in the adult (Fig. 2). There are clearly factors other than ceramide responsible for apoptosis after UUO in the adult. These include altered expression of growth factors such as transforming growth factor-β1, epidermal growth factor, and tumor necrosis factor (14, 23). The early burst in renal apoptosis after UUO in the neonate, which is also independent of changes in renal ceramide content, may relate at least in part to the increased activity of the renin-angiotensin system and a preponderance of AT₂ receptors. We have demonstrated that endogenous angiotensin II contributes to renal apoptosis after 3 days of UUO in the neonatal rat (11). Moreover, administration of exogenous angiotensin II increases renal apoptosis even in the normal neonatal kidney (11). There appears to be a window of susceptibility (from 14 to 28 days of age) for the maturing kidney to increase ceramide production as a consequence of UUO. This increase, in turn, is associated with increased apoptosis compared with the adult. Future studies will be required to determine which factors override the effects of endogenous ceramide on apoptosis in the obstructed kidney during nephrogenesis (through the first 10 postnatal days in the rat).

In summary, we have shown that in response to UUO in the neonatal rat, renal ceramide content (which normally decreases with maturation) increases progressively with 2–4 wk of persistent obstruction. This is associated with a parallel increase in apoptosis in the obstructed kidney. Relief of UUO after 5 days significantly reduces the ceramide content of the post-obstructed kidney. In contrast, UUO has no effect on the ceramide content of the adult kidney. These results are consistent with a role for endogenous ceramide in the enhanced apoptotic response of the neonatal kidney subjected to prolonged UUO.

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