Effect of Unilateral Ureteral Obstruction and Anti-angiotensin II Treatment on Renal Tubule and Interstitial Cell Apoptosis in Rats

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Aim To investigate the effects of angiotensin-converting enzyme inhibitor (cilazapril) and angiotensin II type I receptor antagonist (losartan) on tubular and interstitial cell apoptosis and caspase-3 activity in rats with obstructive nephropathy after unilateral ureteral obstruction.

Methods Rats with unilateral obstructive nephropathy and sham-operated rats were treated with cilazapril, losartan, or the vehicle (water). Tubular and interstitial cell apoptosis was detected morphologically on hematoxylin and eosin-stained renal specimens and by the terminal deoxynucleotidyl transferase-mediated nick end-labeling. Caspase-3 activity in whole-kidney tissue homogenates was measured colorimetrically.

Results After unilateral ureter ligation, there was a significant increase in the number of apoptotic tubular and interstitial cells in the obstructed kidney (13.17 ± 8.73 vs. 3.00 ± 4.53 cells per high power field; $P = 0.049$ and 6.33 ± 3.27 vs 2.00 ± 2.35 cells per high power field; $P = 0.036$, respectively, vs sham-operated rats, 10 days after ligation). In rats with unilateral obstructive nephropathy, neither cilazapril nor losartan had an effect on tubular cell apoptosis. However, cilazapril caused a significant increase in the number of renal apoptotic interstitial cells (7.00 ± 9.74 vs 0.8 ± 1.41 cells per high power field, $P = 0.019$). Caspase-3 activity was not significantly different in rats with unilateral obstructive nephropathy than in sham-operated rats.

Conclusion Rats with unilateral obstructive nephropathy had increased apoptosis of tubular and interstitial cells in comparison with sham-operated rats. Neither cilazapril nor losartan had an effect on tubular cell apoptosis, and cilazapril even increased interstitial cell apoptosis.

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Unilateral ureteral obstruction is a procedure that leads to a number of pathophysiological and morphological changes, including tubular atrophy, interstitial inflammation and fibrosis, and apoptosis of renal tubular and interstitial cells (1), which results in chronic obstructive nephropathy (2). Although apoptosis of renal tubule and interstitial cells is a prominent feature of unilateral obstructive nephropathy, the mechanisms involved in it have not been fully elucidated (3). Recent research has indicated that in unilateral obstructive nephropathy there is an association between the renin-angiotensin system and apoptotic alterations in the kidney (4).

All the components of the renin-angiotensin system are present within the kidney (5), where both classic and alternate pathways are operational. The biological effect of angiotensin II is mediated by cell surface receptors, which can be divided into two main pharmacological classes, angiotensin II receptor subtypes I (AT1) (6) and angiotensin II receptor subtypes II (AT2) (7). The AT1 receptors are responsible for the major actions of angiotensin II, whereas the role of AT2 receptors is still not completely known (8,9). Angiotensin II may induce renal cell apoptosis by promoting oxidative stress, by causing vasoconstriction, and by enhancing the expression of adhesion molecules inducing chemotaxis and cytokine synthesis. In obstructive nephropathy, angiotensin II increases the expression of various factors, including transforming growth factor β1, tumor necrosis factor α (10), platelet derived growth factor, insulin-like growth factor, osteopontin, vascular cell adhesion molecule-1, monocyte chemotactic protein-1, intercellular adhesion molecule-1, and nuclear factor kappa-B (3).

The process of apoptosis is a complex mechanism in which a major role is played by caspases (cysteinyl aspartate-specific proteinase) (11). Many apoptosis-inducing factors (12) transport the signals through the cytoplasm via mediating molecules. These signals are transduced through cytosol by an ever-increasing number of mediator molecules that belong to distinct families (13), each of which mediates a specific apoptotic pathway (14). These pathways, however, converge into a common arm, characterized by an orderly activation of caspases (15), which serve as effector molecules for apoptosis (16,17). One of the best studied effector caspases is caspase-3, the central molecule at the crossroad of all known apoptotic pathways (18).

Although the role of angiotensin II in the pathophysiology of unilateral obstructive nephropathy is clear, there is a shortage of studies comparing the effects of angiotensin-converting enzyme (ACE) inhibition and AT1 antagonism on renal tubule and interstitial cell apoptosis. We hypothesized that both ACE inhibitor (cilazapril) and AT1 receptor antagonist (losartan) would decrease renal tubular and interstitial apoptosis. Also, we expected that cilazapril would have greater antiapoptotic effect than losartan, because of the well-known association between ACE inhibition and increased nitric oxide (NO) generation (9). The potential pharmacologic difference between the two classes of anti-angiotensin drugs with different effects on renal cell apoptosis may have clinical therapeutic implications for patients with obstructive nephropathy.

Materials and methods

The experiments were performed using 2-3-month-old male Wistar rats weighing 210-300 g, at the Department of Physiology, Zagreb University School of Medicine, Zagreb, Croatia.

Unilateral obstructive nephropathy

Rats were divided into two groups, each consisting of 6 animals. Animals were anesthe-

tized before they underwent midline abdominal incision and the left ureter was exposed and ligated using Prolene 4-0 (Ethicon, Johnson & Johnson, Hamburg, Germany) at the transition of the proximal to distal ureter. The sham-operation was performed in the same way, except that after the preparation and visualization of the left ureter no ligature was placed. The kidneys of the sham-operated rats and the contralateral (unobstructed) kidney of the rats with unilateral ureter ligation served as the control. The time course of unilateral obstructive nephropathy was established by killing the rats 4 and 10 days after the surgery. Since apoptosis of tubule and interstitial cell was more pronounced at day 10, all subsequent experiments were performed at day 10.

Treatment with cilazapril or losartan

Both the animals with unilateral obstructive nephropathy and sham-operated animals were treated with water (5 mL orally per day), cilazapril (10 mg/kg orally per day), or losartan (30 mg/kg orally per day).

Cilazapril and losartan were dissolved in water and were administered by gavages. The doses of cilazapril and losartan were chosen according to the literature (19-21). Rats were killed at day 10 after surgery by ether anesthesia. After both kidneys were removed and cut in the sagittal plane, a half of each kidney was fixed in 4% buffered formalin and the other half was stored in saline at -70°C.

Histological analysis

Following fixation in 4% buffered formalin, renal tissue was embedded in paraffin wax and subsequently cut in 3-5 μm-microtome sections. The tissue sections obtained in this way were stained by hematoxylin and eosin. For detection of apoptosis, a subset of kidney tissue slides was labeled by the terminal deoxynucleotidyl transferase-mediated nick end-labeling method (TUNEL, TACS XL kit, R&D Systems Inc. Minneapolis, MN, USA) (Figure 1). Processing of tissue samples was done at Pliva Research Institute, Zagreb, Croatia. Histological examination of the kidney samples was performed by a pathologist unaware of the investigation protocol, who inspected 10 visual fields at a magnification of ×400. The histological samples were analyzed as follows: the extent of apoptosis in renal tubular and interstitial cells was observed on the basis of the characteristic morphological changes in the preparation stained with hematoxylin and eosin. To confirm the results obtained by morphological determination of apoptosis, a subset of slides was processed by TUNEL, and correlation analysis with morphology was performed. Because of an excellent correlation ($r = 0.9$) between morphology and TUNEL, the subsequent determination of apoptosis was done by cell morphology. The average number of apoptotic cells on 10 high power fields (hpf) on a single slide was used in the analysis.

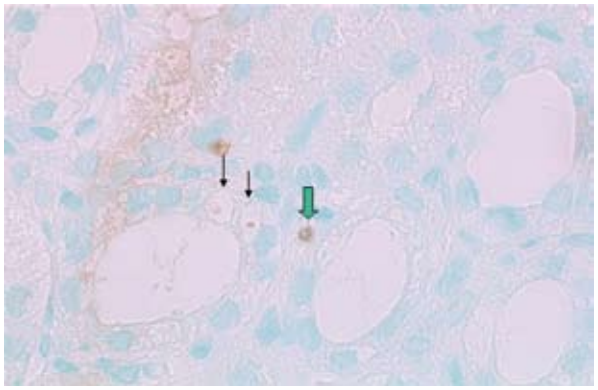

Figure 1. Renal cell apoptosis after 10 days of unilateral obstruction. Black arrow shows apoptotic tubular cells and green arrows show apoptotic interstitial cells in the renal cortex (TUNEL × 400).

Caspase-3 measurement

To measure caspase-3 activity, one sagittal half of the kidney was homogenized, left in cell lysis buffer (50 mmol/L HEPES, pH 7.4, 5 mmol/L CHAPS, 5 mmol/L DTT), and centrifugated (14 000 g/20 minutes). Measurement was performed with a commercial kit according to

manufacturer's instructions (Sigma, Vienna, Austria). The measurement is based on colorimetric determination of p-nitroanilin (pNA) at a wavelength (λ) of 405 nm, as a result of the effects of caspase-3 on its substrate acetyl-Asp-Glu-Val-Asp p-nitroanilid (Ac-DEVD-pNA). The caspase-3 activity was presented as the difference in absorbance per minute per milligram of protein, with and without inhibitor (Ac-DEVD-CHO) (ΔA/mg prot/min).

Statistical analysis

The results are presented as mean ± standard deviations. Statistical significance of the difference between the groups was tested by one-way analysis of variance (ANOVA) with *post-hoc* Newman-Keuls test for multiple comparisons. The statistical analyses were performed with Statistica for Windows v7.1 software (StatSoft, Tulsa, OK, USA). A value of $P < 0.05$ was considered to be statistically significant.

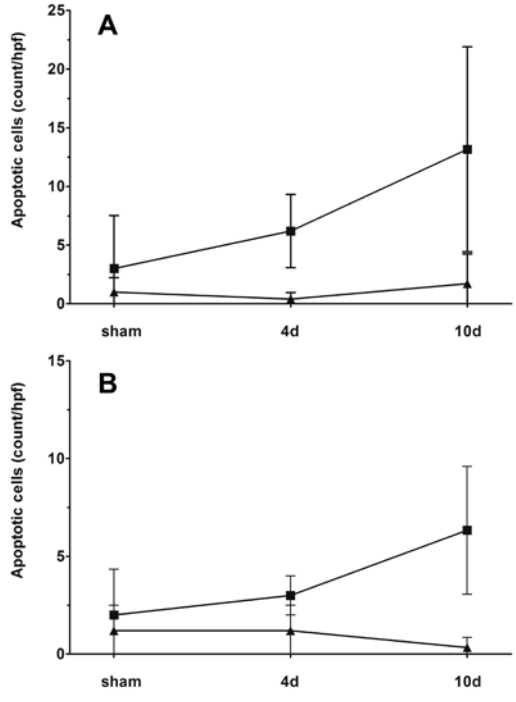

Figure 2. Time course of apoptosis in the kidney during unilateral obstructive nephropathy. Squares – unilateral obstructive nephropathy; triangles – contralateral kidney, d – days. (**A**) tubule cell apoptosis (10-day vs sham, $P = 0.049$; ANOVA with post-hoc Newman-Keuls test); (**B**) interstitial cell apoptosis. (10-day vs sham, $P = 0.036$; ANOVA with post-hoc Newman-Keuls test).

Results

Unilateral ureteral obstruction resulted in time-dependent increase in both tubule and interstitial cell apoptosis in the obstructed kidney (Figure 2A and 2B). In the contralateral (unobstructed) kidney, the number of tubular and interstitial cells apoptotic cells was low and there were no significant changes 10 days after surgery (Figure 2A and B).

In the second part of the study, experimental animals were administered cilazapril, losartan or vehicle (water). In comparison with sham-operated animals (2.27 ± 2.79 cells per field), tubule cell apoptosis increased in animals with unilateral obstructive nephropathy treated with vehicle (23.67 ± 19.27 cells per field; $P = 0.046$), cilazapril (22.50 ± 24.92; $P = 0.037$), and losartan (21.20 ± 30.51; $P = 0.020$) (Figure 3). Similarly, interstitial cells apoptosis also increased in animals with unilateral obstructive nephropathy treated with all three substances, but it was significant only in cilazapril-treated animals (7.00 ± 9.74

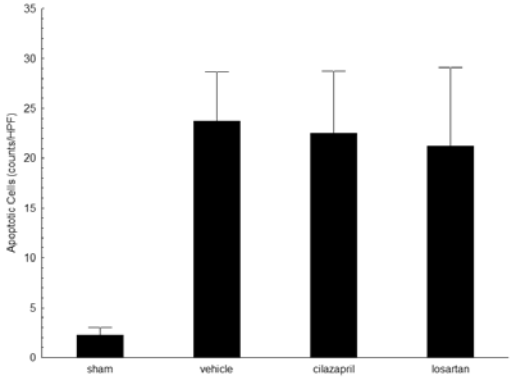

Figure 3. Frequency of apoptotic tubular cells in experimental animals treated with cilazapril (n = 16), losartan (n = 15), or vehicle (water; n = 15) after unilateral obstructive nephropathy or sham operation (n = 15). Statistical differences were tested with ANOVA with post-hoc Newman-Keuls test. In comparison with sham unilateral ureter obstruction, there was a significant increase in tubule cell apoptosis following unilateral obstructive nephropathy in rats treated with vehicle ($P = 0.046$), cilazapril ($P = 0.037$), or losartan ($P = 0.020$).

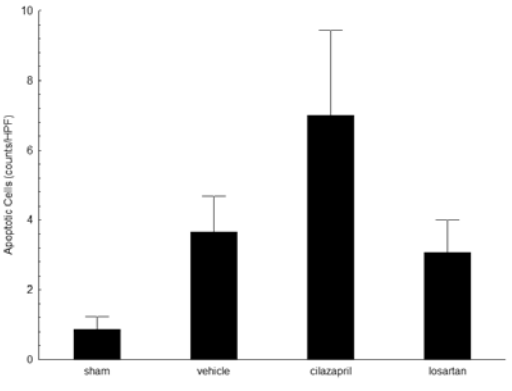

Figure 4. Number of apoptotic interstitial cells in experimental animals treated with cilazapril (n = 16), losartan (n = 15), or vehicle (water; n = 18) following unilateral obstructive nephropathy, or sham obstruction (n = 15). Statistical differences were tested with ANOVA with post-hoc Newman-Keuls test. In comparison with sham unilateral ureter obstruction, there was a significant increase in tubule cell apoptosis following unilateral obstructive nephropathy only in rats treated with cilazapril (P = 0.019).

vs 0.8 ± 1.41 cells per field; $P = 0.019$) (Figure 4). Neither tubular nor interstitial cell apoptosis increased significantly in sham-ligated animals, irrespective of the treatment.

We observed only a trend for an increase in caspase-3 activity in vehicle-treated rats with unilateral obstructive nephropathy (0.0475 ± 0.0449 ΔA/mg prot/min), as compared with sham-operated vehicle-treated, cilazapril-treated, or losartan-treated rats (0.0223 ± 0.0214, 0.0334 ± 0.0188, 0.0246 ± 0.0039 ΔA/mg prot/min, respectively; $P = 0.518$, ANOVA; Figure 5). In the group of sham-ligated experimental animals

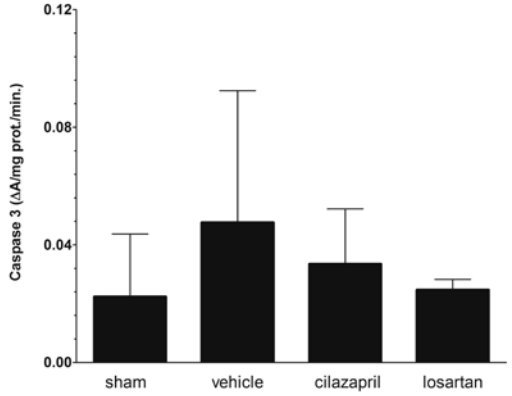

Figure 5. Caspase-3 activity in obstructed kidneys obtained from rats treated with cilazapril, losartan, or water. as a vehicle.

treated with cilazapril, losartan, or vehicle, there was no significant difference in caspase-3 activity between the left and right kidney (data not shown).

Discussion

We demonstrated increased tubule and interstitial cell apoptosis in rats with unilateral obstructive nephropathy. We also showed that anti-angiotensin treatment with either cilazapril or losartan did not decrease apoptosis. Moreover, cilazapril somewhat increased apoptosis in the interstitium of obstructed kidneys. This is not in accordance with recent findings that ACE inhibitor or angiotensin receptor blockade results in decreased interstitial fibrosis, apoptosis, and macrophage infiltration in unilateral obstructive nephropathy (22-24).

During unilateral obstructive nephropathy, both tubule and interstitial cells in the kidney undergo apoptosis (18,25,26). In tubule cells, it is the main mechanism of enhanced cell loss (18), while in interstitial cells, it presumably slows down the development of renal scarring. Therefore, the finding of selective mechanisms leading to apoptosis of renal tubular and interstitial cells would enable novel pharmacotherapeutic approaches for slowing down the progression of chronic obstructive nephropathy. Various studies investigating the effect of inhibition of type 1 and type 2 angiotensin II receptors on apoptosis and renal cell proliferation in unilateral obstructive nephropathy in rats obtained contradictory results (27,28). Pro-apoptotic role of angiotensin in the cardiovascular system via AT2 receptors has been well recognized (29). Beside their antagonistic effect on the vascular system, angiotensin II and NO may also act antagonistically on cell apoptosis (30).

The present study showed that unilateral obstructive nephropathy was associated with a

significant increase in the number of apoptotic renal tubular and interstitial cells, which is consistent with previous findings (3,18). The lack of effect of cilazapril and losartan on renal cell apoptosis may not be in agreement with some previous findings, where an ACE inhibitor, enalapril, or an AT1 receptor antagonist, losartan, decreased FasL expression in obstructed kidney, suggesting an antiapoptotic effect of antiangiotensin treatment (21). However, that study did not report the extent of apoptosis in the kidney. Our results corroborate the findings of Yang et al (31), who showed that losartan did not affect renal cell apoptosis in mice with unilateral obstructive nephropathy. Thus, our study complements their findings by investigating apoptosis in another animal model (rat) and in two separate kidney parts – tubules and the interstitium.

We found a significantly higher increase in the number of apoptotic interstitial cells in animals with unilateral obstructive nephropathy treated with cilazapril than in animals treated with vehicle. Similarly, Miyajima et al (32) showed a large increase in the number of apoptotic and proliferative tubular cells in inducible-NO synthase gene knockout mice after unilateral obstructive nephropathy, which was indicative of anti-apoptotic properties of NO. ACE inhibitors prevent degradation of bradykinin, which in turn increases bradykinin-induced generation of NO by endothelial NO synthase. However, contrary to the results of Miyajima et al, we failed to show an enhanced anti-apoptotic effect of cilazapril in the tubular cells and even received a somewhat increased pro-apoptotic effect in interstitial cells. This result may be due to different pathophysiological role of NO generated by the inducible as opposed to endothelial isoforms of NO synthase. To address the exact mechanisms by which ACE inhibitors may promote interstitial cell apoptosis, further investigation is required.

Animals with left ureter obstruction treated with cilazapril, losartan, or vehicle showed only a trend of increase in caspase-3 activity. Also, there were no significant differences in caspase-3 activity between the experimental groups. This discrepancy between caspase-3 activity and apoptosis may be due to different sampling for histology and caspase measurement. Namely, caspase-3 activity was measured in whole kidney homogenates, while apoptosis was determined only in the cortex.

A recent study by Yang et al (31) has demonstrated that hepatocyte growth factor (HGF), a multifunctional protein with potent renotropic properties, may have therapeutic effects preventing chronic renal fibrosis. It has shown that the combination of HGF gene therapy with inhibition of the renin-angiotensin system produced synergistic beneficial effects, leading to a dramatic attenuation of renal tubulointerstitial fibrosis in obstructive nephropathy in mice. The combined treatment with human HGF gene and losartan preserved the renal mass and gross morphology of the obstructed kidneys. Although losartan alone only marginally inhibited apoptosis in obstructed kidneys, the combination of HGF and losartan dramatically prevented cell death. Thus, solely aiming at the renin-angiotensin system by pharmacologic inhibition of ACE or AT1 receptors, one may miss other potential therapeutic targets for the treatment of chronic renal lesions.

Renal cell apoptosis may not occur only during established unilateral obstructive nephropathy, but it may also be a prominent feature of the recovery from unilateral obstructive nephropathy (33). Although we did not specifically address apoptosis during the recovery from unilateral obstructive nephropathy, our results showing enhanced interstitial cell apoptosis with cilazapril corroborate these findings and further point to a possible protective effects of ACE inhibition.

Protective effects of ACE inhibition on kidney injury may be age-dependent, because in neonatal rats with partial ureter obstruction, enalapril worsened indices of kidney injury, even when administered after postnatal nephrogenesis had been completed (34).

In summary, the present study shows that unilateral obstructive nephropathy in rats is associated with an increase in renal tubule and interstitial cell apoptosis. Because only cilazapril, as an ACE inhibitor, was associated with increased interstitial cell apoptosis (as compared with vehicle and losartan) without significantly influencing tubule cell apoptosis, we believe that ACE inhibitors may be considered first-choice therapy for patients with chronic obstructive nephropathy. However, additional studies are necessary to test this hypothesis prospectively in animals or humans.

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