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ORIGINAL PAPER

Antiapoptotic effect of angiotensin-II type-1 receptor blockade in renal tubular cells of hyperoxaluric rats

Matem Tunçdemir · Oktay Demirkesen · Melek Öztürk · Pınar Atukeren · M. Koray Gümüştaş · Tahir Turan

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Abstract In this study, we investigated the protective effect of losartan as an AT1 receptor antagonist by evaluating the expression of apoptosis-regulatory genes that contribute to the progressive damage in the renal tubules of hyperoxaluric rats. Rats were divided into 4 groups of 10 each; control (C), ethylene glycol (EG), ethylene glycol + losartan (EG + L) and Losartan (L). For 4 weeks 0.8% EG, as a precursor for oxalate, was administered to EG and EG + L and losartan (300 mg/l) was administered to groups EG + L and L. Urine and blood samples were collected for biochemical determination. Bcl-2, bax, caspase-3 and TGF-beta 1 antibodies were used for immunohistochemistry. Apoptosis was determined by TUNEL method. A marked increase in urinary oxalate levels of the rats in EG and EG + L groups was found. In the EG group a diffuse amount of oxalate crystals into the tubular lumina and interstitium in the cortex was observed. In the EG group GBM thickening, interstitial fibrosis and tubular atrophy with infiltration of mononuclear cell findings reduced in the EG + L group were presented as well. In the EG group, immunoreactivity of TGF-beta 1 was increased in glomeruli and tubuli. In the EG + L group, immunoreactivity of TGF-beta 1 was decreased compared to the EG group. Bax expression increased in the renal tubules of EG group and reduced in the EG + L group comparing to the control. In the EG + L group, the immunoreactivity of bcl-2 was increased in glomeruli. In EG + L treated group, number of caspase-3 immunopositive cells were decreased compared to all groups ($P < 0.01$). Apoptotic cells were increased in the EG-treated group compared to the other groups. Decreased apoptotic cell number was observed in the EG + L compared to the EG group ($P < 0.01$). Our findings suggest that losartan may provide a beneficial effect against tubulointerstitial damage and decrease renal tubular apoptosis caused by hyperoxaluria.

Keywords Hyperoxaluria · Apoptosis · Rat · Kidney · AT1 receptor blocker

M. Tunçdemir · M. Öztürk
Department of Medical Biology,
Cerrahpasa Faculty of Medicine, Istanbul University,
Istanbul, Turkey

O. Demirkesen
Department of Urology, Cerrahpasa Faculty of Medicine,
Istanbul University, Istanbul, Turkey

P. Atukeren · M. K. Gümüştaş
Department of Biochemistry, Cerrahpasa Faculty of Medicine,
Istanbul University, Istanbul, Turkey

T. Turan (✉)
Department of Urology, Faculty of Medicine,
Pamukkale University, Denizli, Turkey
e-mail: tturan@pau.edu.tr

Introduction

Hyperoxaluria (HOX) is a well-known cause of renal stone disease [1] and tubulointerstitial (TI) damage [2–4]. It has been shown that high levels of urinary oxalate cause renal epithelial cell injury and crystal retention [5]. The smaller crystals are endocytosed, whereas the larger ones may be overgrown by tubular epithelium and so incorporated into the interstitium, where it leads to TI inflammation [2, 6]. HOX and crystal-mediated cellular response seem to play an important role in the pathogenesis of urolithiasis.

Oxalate exposure triggers both necrotic and apoptotic cell death in renal epithelium. Oxalate-induced death of renal epithelial cells exhibits several features characteristic of apoptosis, including abundance of apoptotic bodies,

increased production of ceramide and marked sensitivity to the level of expression of the antiapoptotic genes [5, 7–9]. Oxalate-induced cell death demonstrates several characteristics of necrosis, in that the majority of the cells exhibited cellular and nuclear swelling after oxalate treatment and showed little evidence of DNA cleavage by TUNEL assay [5, 10]. It was also reported that the expression of various cytokines, chemokines and other mediators of inflammatory response, including angiotensin II (AII) have been demonstrated in renal tubular cells [11, 12]. Local AII seems to play an important role in the majority of glomerular and TI disease by inducing or amplifying the inflammatory response independent of etiology. The renin–angiotensin system (RAS) was pointed out to be significant in the development of TI damage in several models of chronic TI injury and angiotensin II type 1 receptor (AT1R) stimulation was assumed to trigger the pro-fibrotic mechanism [13]. It has been shown that AII stimulates the synthesis of transforming growth factor-beta 1 (TGF-beta 1), produced mainly in damaged tubular epithelial cells and in macrophages [14]. TGF-beta 1 induces apoptosis in the tubular epithelial cells and contributes to progressive tubular atrophy [15]. Angiotensin-converting enzyme (ACE) inhibitors and AT1R blockers have been reported to protect against interstitial damage in different TI lesion models regardless of their effect on blood pressure [13, 16–18]. Losartan and its principal active metabolite block the vasoconstrictor and aldosterone-secreting effects of angiotensin II by selectively blocking the binding of angiotensin II to the AT1 receptor found in many tissues, (e.g., vascular smooth muscle, adrenal gland). Both losartan and its principal active metabolite do not exhibit any partial agonist activity at the AT1 receptor and have much greater affinity (about 1,000-fold) for the AT1 receptor than for the AT2 receptor. Neither losartan nor its active metabolite inhibits ACE (kininase II, the enzyme that converts angiotensin I to AII and degrades bradykinin); nor do they bind to or block other hormone receptors or ion channels known to be important in cardiovascular regulation.

Using this experimental model of ethylene glycol-induced hyperoxaluria we evaluated the effect of AT1R blockade by losartan on renal epithelial apoptosis in hyperoxaluric rats. To our knowledge this is the first report evaluating the effects of an AT1R blocker on apoptosis of renal epithelial cell caused by hyperoxaluria in vivo.

Materials and methods

Animals and protocols

All experiments were approved by the Hospital Ethics Committee for Animal Experiments (no: 2003TPF 008) and followed the NIH Guide for the Care and Use of Laboratory Animals. Male Wistar rats initially weighing 250–290 g. were housed in metabolic cages at room temperature with a 12-h light–darkness cycle. After 7 days they were divided into 4 groups of 10 each, namely control (C), ethylene glycol (EG), ethylene glycol + losartan (EG + L) and losartan (L) group. All animals had access to tap water and free access to standard rat chow. For 4 weeks 0.8% ethylene glycol (to EG and EG + L) and losartan 300 mg/l (to EG + L and L) were continuously administered in drinking water. We collected 24-h urine samples for biochemical determination at baseline and after 4 weeks together with systolic blood pressure values measured by tail cuff plethysmography.

All animals were killed after 4 weeks. The kidneys were perfused with saline solution through the abdominal aorta before killing until it was free of blood. Formalin fixed and paraffin-embedded renal tissue sections were histologically scored for tissue damage and immunohistochemically stained for the evaluation of TGF-beta 1, proapoptotic (Bax), antiapoptotic (Bcl-2) and effector caspase (caspase-3) protein expression. Apoptotic changes were evaluated by using the TUNEL method. For light microscopy, the rat kidney sections were stained with hematoxylin and eosin and periodic acid-Schiff reagent.

Biochemical procedures

Urinary creatinine levels were measured by the enzymatic kit (Teco Diagnostic, USA) depending on Jaffe's method. Urine oxalate levels were assayed by the enzymatic method (Sigma Chemical Co., St. Luis, Missouri) and urine PH was determined by using strips for urinanalysis (URS-10, Teco Diagnostic, USA).Urinary albumin concentration was measured by the radial immunodiffusion method.

Histological evaluation

For light microscopy, the rat kidney slices were stained with hematoxylin and eosin and periodic acid-Schiff reagent. Tubular atrophy, infiltration of mononuclear cells (MNC), interstitial fibrosis and crystal deposition (polarized light) were analyzed in 10 microscopic fields per section by using 40× objective and scored according to the scale 0 = no damage; 0.5 = weak damage (involving ≤25% of each microscopic field); 1 = moderate damage (>26% and ≤50%); 2 = severe damage (>51% and ≤75%); 3 = very severe damage (>76% of the field). We also counted the fraction of damaged glomeruli with glomerular basement membrane (GBM) thickening on the total amount of glomeruli in three random non-overlapping places. Two

observers carried out all examinations blinded to the treatment group.

Immunohistochemistry

The renal tissue sections were placed onto slides coated with poly-L-Lysine (PLL, 0.1% w/v in water, Sigma, St. Louis, MO) and were left overnight to dry at 37°C then deparaffinized and rehydrated. Immunoperoxidase staining was performed using Novostain Universal Detection Kit NCL-RTU-D (Novo Castra Lab, UK), Anti-Rabbit HRP/AEC (TR–015-HA), Ultra Vision Antibody Detection System (LabVision Corp, USA) kits. Immunostaining procedures were carried out following the guidelines of the manufacturer. After a washing step in phosphate buffered saline (PBS), sections were incubated for 10 min. with 3% H_2O_2 in PBS to block endogenous peroxidase activity. After the sections were washed with PBS, incubation with normal blocking serum was performed. Sections were incubated with mouse monoclonal Bcl-2 (Santa Cruz Biotech. sc-7382, 1:100 dilution), mouse monoclonal Bax (Santa Cruz Biotech. sc-7480, 1:100 dilution), rabbit polyclonal caspase-3 (NeoMarkers, CPP32, 1:100 dilution) and TGF-beta 1 (Santa Cruz Biotech. sc-146, 1:200 dilution) antibodies at room temperature for 1 h, followed by PBS washing, incubation with biotinylated secondary antibody, and a final PBS washing. Then sections were incubated with a substrate–chromogen solution (AEC; Zymed, San Francisco CA, USA) for 5–6 min before another incubation with horseradish peroxidase–streptavidin complex. Sections were counterstained with hematoxylin.

To determine the specificity of immunostaining, sections were incubated following the above procedure, except for incubation with primary antibody. Instead of primary antibody, control serum was also used as control.

Evaluation of the immunostaining

Semiquantitation of immunoperoxidase staining

Immunoperoxidase staining of glomeruli, tubules and interstitial area was analyzed, and scored from as no staining (–), weak (+), moderate (++) and strong (+++). The blinded and randomized mode of analysis was performed at ×40 magnification for all immunoperoxidase stained sections in a minimal of 10 fractions per kidney section as previously described [19, 20].

Quantitation of caspase-3 immunoreaction

The number of caspase-3-immunoreactive cells was analyzed in 10 fractions per rat at a magnification of ×40. All animals were included in the data analyzed.

TUNEL

Kidney tissues were fixed in 10% neutral buffered formalin, embedded in paraffin wax, and then cut into 5-μm thick sections. Sections were put on slides coated with poly-L-lysine (PLL; Sigma). DNA fragmentation for apoptosis was detected by the modified TUNEL method. TUNEL staining was performed using an apoptosis detection kit (in situ cell death detection kit, horse-radish peroxidase (POD); Roche Diagnostics GmbH, Mannheim, Germany). The sections were incubated with proteinase K (20 μg/ml) for 15 min at 37°C. Incubating the sections with 0.3% H_2O_2 in methanol for 10 min followed by rinsing 3 times with PBS for 2 min quenched the peroxidase. Sections were then incubated with the TUNEL reaction mixture (TdT mediated dUTP nick end labeling solution, 1.10 diluted) at 37°C in a humid chamber for 60 min, which was again followed by rinsing with PBS. The sections were incubated with Converter-POD (fab fragment from sheep, conjugated with horseradish peroxidase) at 37°C in a humid chamber for 30 min and rinsed with PBS. The sections were then treated with 1× DAB-substrate diluted with POD buffer and the reaction was allowed to run at room temperature for 5 min. Cells with brown nuclear labeling were defined as TUNEL-positive. Staining was evaluated using a light microscope after counterstaining with methyl green.

Staining specificity controls

Thymus tissue sections from dexamethasone-treated rats (5 mg/kg, i.p.) were used as positive control [21]. For negative controls, distilled water was used instead of TUNEL reaction mixture.

Apoptotic index

Morphometric analysis of the positive cells in tissue stained by TUNEL method was performed under high power magnification (×40) in a blinded fashion. On each slide, 15 fields were randomly selected. To quantitate the extent of apoptosis, we recorded numbers of apoptotic cells (TUNEL-positive cells) in sections from the four groups. We totaled all TUNEL-positive and intact cells in those fields, and then calculated apoptotic index by means of an average count per slide. Apoptotic index was calculated according to the formula: $AI = (AC/AC + IC) \times 100$, (AI Apoptotic index, AC Apoptotic cell number, IC Intact cell number) [22].

Statistical analysis

The values of urinary creatinine, oxalate, albumin, urine pH, caspase-3-immunoreactive cell numbers and apoptotic

index were expressed as mean ± SD. Kruskall–Wallis, ANOVA and Wilcoxan rank tests were used and a value of $P < 0.05$ was considered as significant. Statistical analysis for caspase-3 immunoreaction, after the normality of variables was checked, a Kruskall–Wallis test was used and subsequently Mann–Whitney *U* non-parametric test was performed for significant data obtained in the Kruskall–Wallis test.

Results

Biochemical results

There were no significant differences in the baseline values of systolic blood pressure, urinary pH, urinary albumin, oxalate and creatinine excretion among the groups. Average daily water intake throughout the 4 weeks in groups 1 to 4 was 28.3 ± 0.6, 27.3 ± 1, 28.1 ± 0.7 and 27.8 ± 0.8 ml, respectively (*P* not significant). At the end of the study, biochemical analysis revealed a marked increase in urinary oxalate levels of the rats in EG group and EG + L group ($P < 0.05$), while decreased creatinine excretion was detected only in the EG group ($P < 0.01$). No significant changes in systolic blood pressure, urinary pH and urinary albumin levels were observed in any group at the end of the study (Table 1).

Light microscopy

In the light microscope we observed that animals in ethylene glycol treated group had a diffuse amount of oxalate crystals into the tubular lumina and interstitium, mainly in the cortex. In addition, GBM thickening, interstitial fibrosis and tubular atrophy with MNC infiltration in the cortex and medulla were also present in the EG group. These findings reduced in the ethylene glycol animals treated with losartan, but were not completely normalized (Table 2). Although there were no differences in urinary oxalate levels between the EG group and EG + L group, it was observed that losartan treated ethylene glycol group had different and better morphological scores than ethylene glycol treated group.

Immunohistochemistry

Transforming growth factor-beta 1 (TGF-beta 1)

The immunoreactivity of TGF-beta 1 was rarely observed in the glomeruli and interstitium and was not observed in the tubules of the control group (Fig. 1a-A). In the EG group, immunoreactivity of TGF-beta 1 was found to markedly increased in glomeruli and tubules (Fig. 1a-B). In the EG + L group, immunoreactivity of TGF-beta 1 was decreased compared to the EG group. TGF-beta 1 immunopositivity was seen especially at the juxta glomerular apparatus in the EG + L group (Fig. 1a-C). In the L group, immunoreactivity of TGF-beta 1 was similar to the control group (Fig. 1a-D; Table 3).

Bax

The weak immunoreactivity of bax was observed in the glomeruli and interstitial areas of the control group (Fig. 1b-A). In the EG group, immunoreactivity of bax increased in the renal tubules compared to the control group (Fig. 1b-B). In the EG + L group, bax immunoreactivity was weaker than EG group especially in the glomeruli and tubules (Fig. 1b-C; Table 3).

Bcl-2

In the control group, bcl-2 immunoreactivity was observed weakly in the glomeruli and interstitial areas and there was no immunoreactivity at tubules (Fig. 1c-A). In the EG + L group (Fig. 1c-D), the intensity of bcl-2 immunoreactivity was stronger in the glomeruli, tubules and interstitial areas compared to EG group (Fig. 1c-B,C) (Table 3).

Table 1 Mean parameters at 4 weeks

	Control ($n = 10$)	Ethylene glycol ($n = 10$)	Ethylene glycol + losartan ($n = 10$)	Losartan ($n = 10$)
Systolic blood pressure(mm Hg)	128.2 ± 4	126.4 ± 6	125.1 ± 5	127.2 ± 5
Urine pH	8.2 ± 1.3	8.3 ± 1	8.2 ± 1.1	8.4 ± 1.2
Urine albumin (mg/day)	3.25 ± 0.17	3.0 ± 0.0	3.0 ± 0.0	3.1 ± 0.1
Urine oxalate (mg/day)	50.03 ± 2.64	100.48 ± 11.64[a]	84.64 ± 12.78[a]	56.64 ± 13.52
Urine creatinine (mg/dl)	7.32 ± 2.42	4.09 ± 0.94[b]	12.05 ± 1.44	9.68 ± 1.83

Values are mean ± SD

[a] $P < 0.05$ versus control and losartan groups

[b] $P < 0.01$ vs all groups

Table 2 Results of histological damage

Group	*n*	GBM thickening (fraction)	Tubule	Tubular atrophy	Crystal deposition	Site	Interstitial fibrosis	MNC infiltration
Control	10	0.054	P	0.0	0.0	CX	0.0	0.1
			D	0.0	0.0	M	0.0	0.1
			C	0.0	0.0			
Ethylene glycol	10	0.288	P	1.6	1.8	CX	2.0	2.1
			D	1.2	1.4	M	1.6	1.2
			C	0.8	0.8			
Ethylene glycol + losartan	10	0.126	P	0.6	0.8	CX	0.6	0.5
			D	0.4	0.6	M	0.4	0.4
			C	0.2	0.2			
Losartan	10	0.076	P	0.1	0.0	CX	0.1	0.1
			D	0.1	0.0	M	0.1	0.1
			C	0.0	0.0			

Tubulointerstitial lesion score: 0 = no damage; 0.5 = weak damage (involving ≤25% of each microscopic field); 1 = moderate damage (>26% and ≤50%); 2 = severe damage (>51% and ≤75%); 3 = very severe damage (>76% of the field)

GBM-thickening = thickened glomeruli/total number of glomeruli

P proximal tubule, *D* distal tubule, *C* collecting duct, *CX* cortex, *M* medulla

MNC mononuclear cells

Caspase-3

A few numbers of caspase-3 immunopositive cells were seen in the control group (Fig. 2a). The number of caspase-3 immunopositive cells was observed especially in the tubule epithelial cells of EG group when compared to the other treated and control groups ($P < 0.01$) (Fig. 2b). In EG + L treated group, numbers of caspase-3 immunopositive cells were significantly decreased compared to all groups ($P < 0.01$) (Fig. 2c; Table 4).

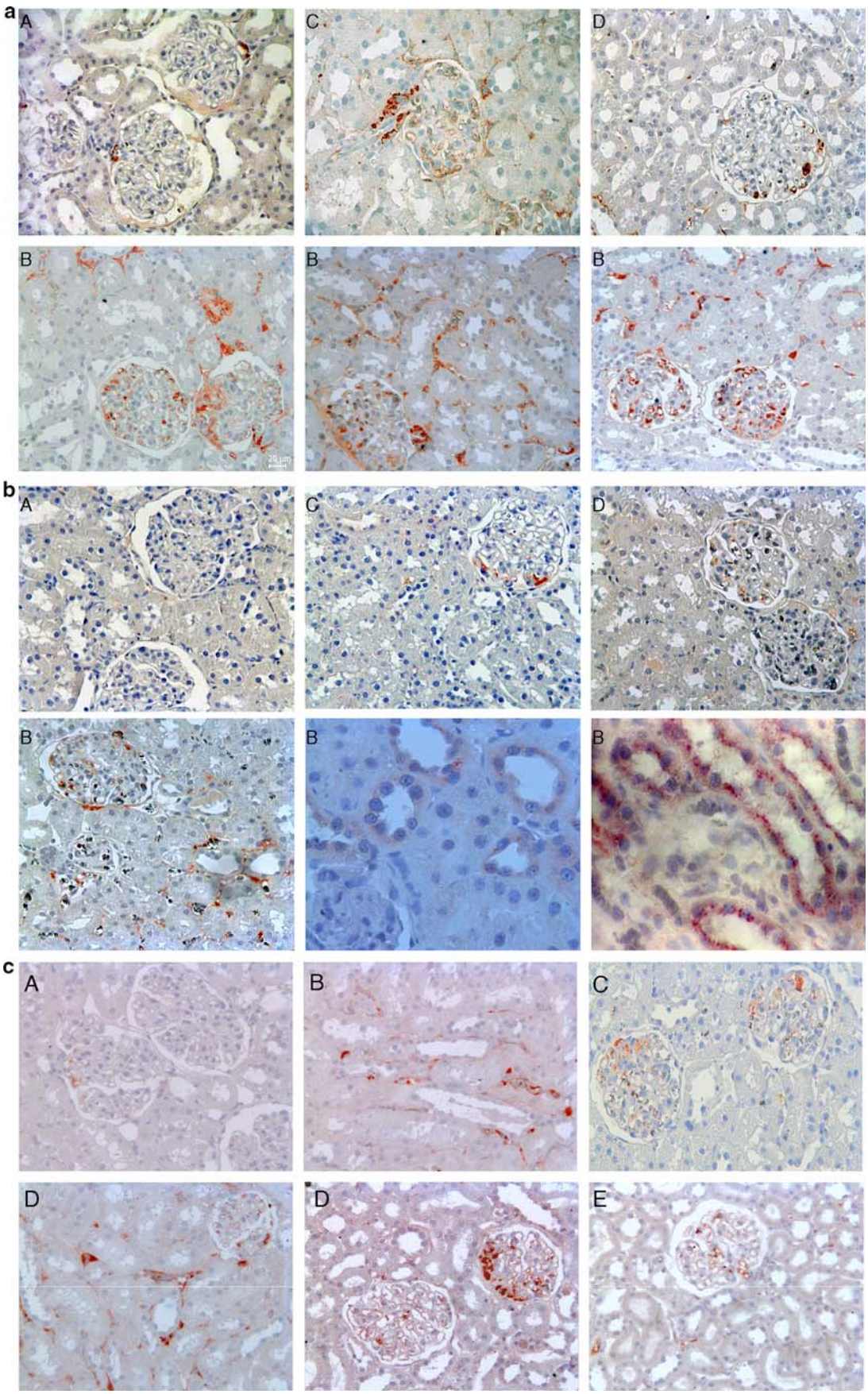
TUNEL

In the control group apoptotic cells were observed rarely (Fig. 3a). In EG group, apoptotic cells were significantly increased in the cortex and medulla ($P < 0.01$) compared to other groups (Fig. 3b). A significant decrease in the number of apoptotic cells was observed in EG + L group ($P < 0.01$) compared to EG group (Fig. 3c). In L group, low number of apoptotic cell was observed (Fig. 3d; Table 4).

Discussion

It is well-known that renal tubular epithelium is the major target for oxalate-induced injury, and CaO_x crystals or oxalate ions play a critical role in the formation of urinary calculi [8]. Oxalate toxicity may also contribute to the observed renal regression in end-stage renal disease, when oxalate reaches toxic levels in the blood and urine [23]. In our study we determined a significant increase of urinary oxalate levels in groups EG and EG + L when compared to the other groups. However, animals only in EG group had a diffuse amount of oxalate crystals into the tubular lumina and interstitium, mainly in the cortex. In addition, GBM thickening, interstitial fibrosis and tubular atrophy with MNC infiltration in the cortex and medulla were also present in EG group. These findings were reduced in group EG + L. Toblli et al. have found similar results and in their study a few crystal deposits were observed in hyperoxaluric rats treated with losartan, although urinary oxalate excretion and urinary calcium oxalate supersaturation were similar to those in the untreated hyperoxaluria group. They also showed that AII type 1 receptor blockade by losartan produces a beneficial effect against renal lesions caused by hyperoxaluria and preserves renal function as well [18]. This fact suggests that interaction with RAS provokes some favorable effect on preventing crystal deposition not only in tubular lumina, but also in the renal interstitium.

CaO_x crystals produce cell damage in proximal tubular epithelium followed by monocyte and macrophage infiltrates. Inflammation contributes to release of AII and TGF-beta 1 from tubular cells, monocytes and macrophages. TGF-beta 1 contributes to transform fibroblasts into myofibroblasts and these cells produce extracellular matrix protein [17]. Fibrosis can lead to edematous swelling of the renal matrix, proliferation of interstitial cells and the induction of an inflammatory reaction. In this study the increase of TGF-beta 1 immunoreactivity in the interstitial regions and tubules in EG group were demonstrated. We determined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◀ **Fig. 1** **a** Immunoreactivity of TGF-beta 1. Control (**A**) and L (**D**) groups. Increased TGF-beta 1 immunoreactivity in the tubules, glomeruli and interstitial areas of EG group (**B**) decreased TGF-beta 1 immunoreactivity in EG + L group (**C**) (×20). **b** Immunoreactivity of Bax. Control (**A**) and L (**D**) groups. Bax immunoreactivity is stronger in the glomeruli and tubules of EG group compared to control group (**B**). In the EG + L group immunoreactivity of bax is seen weaker in glomeruli and tubules compared to EG group (**C**). (A, B1, C ×20, B2, B3 ×100). **c** Immunoreactivity of Bcl-2. Control (**A**) and L (**E**) groups. Bcl-2 immunoreactivity is seen in glomeruli (**B**), tubules and interstitial areas in EG group (**C**), increased immunoreactivity of bcl-2 in the glomeruli, tubules and interstitial areas of the EG + L group (**D**) (×20)

a significant decrease of TGF-beta 1 immunoreactivity in EG + L group compared to EG group.

The induction of apoptosis by oxalate in renal epithelial cells has already been described [9]. The interaction between oxalate and renal epithelial cells elicits a programmed sequence of events that can lead either to cell proliferation or to cell death. Thus, as a distinctive form of cell death, apoptosis could also be responsible for the injury induced by hyperoxaluria. Sarica et al. [8] have suggested that apoptosis in renal tubular cells induced by massive hyperoxaluria might result in cell degradation and may play a role in the pathologic course of urolithiasis. In our study, while rare apoptotic cells were determined at healthy group tubules, there were many apoptotic cells at the dilated tubules of ethylene glycol group. We determined that the increase of apoptotic cell numbers in the kidney tubules supported to the increase of urinary oxalate levels especially in the ethylene glycol group. The numbers of apoptotic cells at tubules of losartan treated ethylene glycol group is decreased in comparison to the ethylene glycol group. These findings suggest that RAS interaction by losartan produces a beneficial effect against renal tubular apoptosis induced by hyperoxaluria.

The control of apoptosis has been related to bcl-2 and bax proteins and activation of apoptosis may depend on the

Table 3 Immunohistochemical distribution of TGF-beta 1, bax and bcl-2 in the kidney tissue sections of all groups

Group	*n*	TGF-beta 1			Bax			Bcl-2		
		Glomeruli	Tubuli	Interstitial area	Glomeruli	Tubuli	Interstitial area	Glomeruli	Tubuli	Interstitial area
Control	10	+	–	+	+	+	+	+	–	+
Ethylene glycol	10	+++	+++	+	++	++	+++	++	+	++
Ethylene glycol + losartan	10	++	+	+	+	+	+	++	++	+++
Losartan	10	+	+	+	+	+	+	+	–	+

– non staining, + weak staining, ++ moderate staining, +++ strong staining

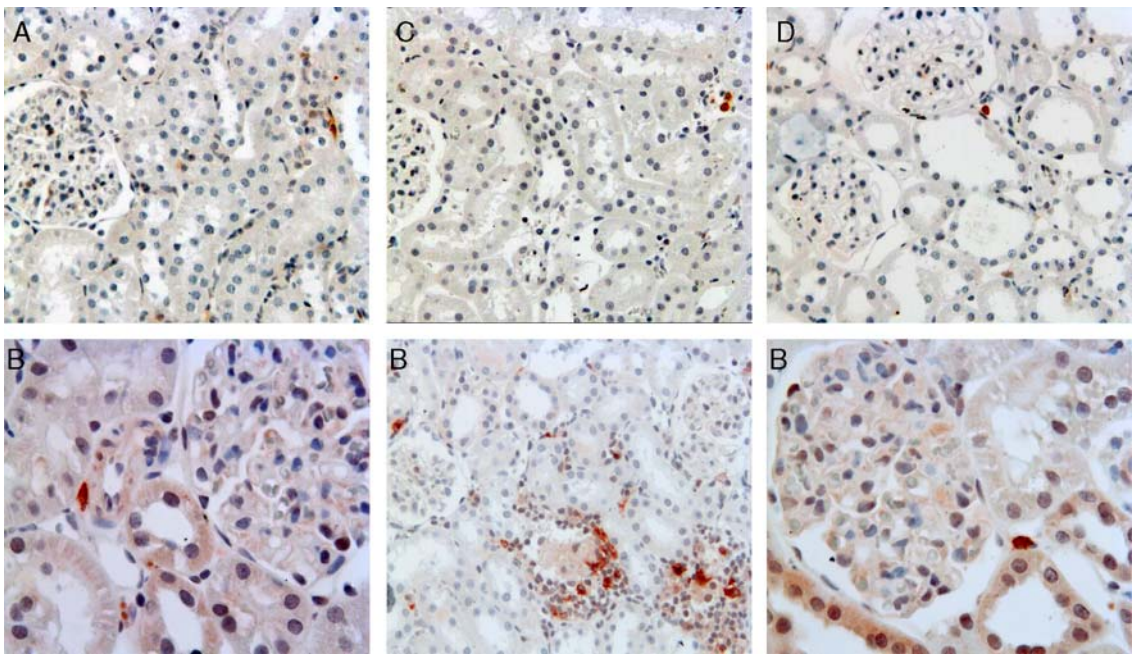

Fig. 2 Immunoreactivity of caspase-3. Control (**A**) and L (**D**) groups. Increased caspase-3 immunoreactivity is seen in renal tubules of EG group (**B**), whereas decreased in the EG + L group (**C**) (A, C, D ×20, B ×40)

Table 4 Evaluation of caspase-3 immunoreaction intensity and apoptotic index of the kidney tissue sections

Group	*n*	Tubular caspase-3	Glomerular caspase-3	Interstitial caspase-3	Apoptotic index
Control	10	13.80 ± 2.17	5.00 ± 1.87	17.4 ± 8.2	1.8 ± 0.92
Ethylene glycol	10	31.20 ± 2.17[a]	9.60 ± 4.39[c]	35.0 ± 10.49[e]	29.6 ± 3.50[f]
Ethylene glycol + losartan	10	5.60 ± 2.88[b]	0.60 ± 0.55[d]	7.6 ± 1.14	17.3 ± 3.00[g]
Losartan	10	14.40 ± 5.50	4.60 ± 3.44	24.2 ± 9.60	7.7 ± 0.30

Values are mean ± SD

[a, f] $P < 0.01$ versus all groups

[b] $P < 0.01$ versus ethylene glycol, control, losartan groups

[c] $P < 0.01$ versus control groups

[d] $P < 0.01$ versus ethylene glycol, losartan groups

[e] $P < 0.01$ versus ethylene glycol + losartan groups

[g] $P < 0.01$ versus ethylene glycol group

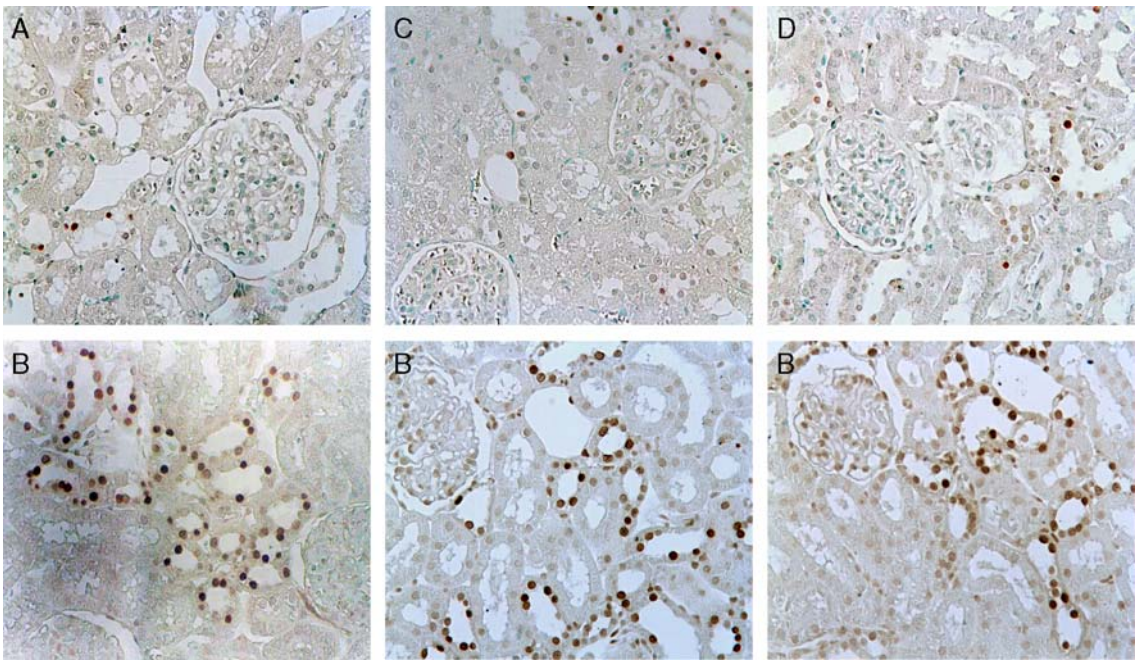

Fig. 3 TUNEL method. Control (**A**) and L (**D**) groups. In the tubules of EG group increased apoptotic cell number was seen (*Brown nuclei*) (**B**). A markedly decreased apoptotic cells in EG + L group (**C**) (×20)

relative balance between bcl-2 and bax [24]. Bcl-2 is known as "the suppressor" and bax appears to play a counterbalancing role by facilitating apoptosis. Over expression of bcl-2 protects from oxalate toxicity and the hold down of oxalate toxicity by bcl-2 is a marker of apoptosis for oxalate induced cell death [5]. Miyazawa et al. [25] demonstrated that the expression of apoptosis-related genes including bax is increased in the kidneys of stone-forming rats. It has also been defined that bcl-2 expression is increased in damaged tubules of rat kidney tissue with bilateral ureteral obstruction [26].

Apoptosis in general is known to proceed initially along distinct pathways, which later converge into a common arm characterized by orderly activation of caspases. Caspase-3 is potentially the most important effector enzyme in apoptosis, providing a common pathway to both death receptor and mitochondria-dependent apoptotic mechanisms. Many death signals including oxidative stress can cause irreversible dysfunction of mitochondria, leading to the release of several mitochondrial intermembrane space proteins, such as cytochrome-*c* into the cytosol, where it initiates caspase activation and apoptosis [27]. It has been shown that caspase-3 may have a central role in renal cell apoptosis and apoptosis through caspase-3 is being stimulated by cytochrome-*c* [28]. Caspase-3 has also been linked to the pathogenesis of

other models of chronic renal injury associated with apoptosis [29].

In this study, we showed that the immunopositivity of bcl-2, bax and caspase-3 was increased in the glomeruli, tubules and interstitial areas in ethylene glycol group in comparison to the healthy controls. In losartan-treated ethylene glycol group, while bcl-2 expression was increased at all areas, the bax and caspase-3 expression were decreased compared to EG group as parallel to the decrease of the apoptotic cell numbers in the tubules.

Several factors, including ischemia, activated cytokines, growth factors, AII and reactive oxygen species (ROS), which are known to induce apoptosis in general, develop during the course of hyperoxaluria and may initiate renal cell apoptosis [8, 10, 18, 25, 30, 31]. Hyperoxaluria causes production of ROS as a result of lipid peroxidation in renal tubular cells, which generally leads to the functional impairment of cellular components and tubular ischemia ensues. Oxidative stress is reported to cause local RAS activation as a result of hyperoxaluria, which is developed in tubular cells due to ROS stimulation [30]. AII increase, which is an effector peptide of RAS, regulates apoptosis, cellular proliferation and fibrosis. It is reported that HOX stimulates the inflammatory response in renal tissue by the activation of RAS, not only by AT1 but also by AT2 receptors of AII. This inflammatory reaction suggested to be mediated by the activation of NF-kappa B and overexpression of cytokines, such as IL-6 and chemokines, such as MCP-1, RANTES. For this reason in the controlling of the inflammation-repairing process in CaO_x crystal injury in renal tissue, blockage of RAS might be required [31]. Increase in *c*-myc gene expression by oxalate induction is reported to play a role in cell proliferation and development of apoptosis. Increased *c*-myc gene expression causes the effects of TGF-beta 1 on cell proliferation and apoptosis [25, 30]. In diabetic and hypertensive rats, RAS interaction produces a substantial benefit by controlling tissue damage and prevents renal [22] and cardiac [32] apoptosis.

Taking the injurious effects of hyperoxaluria-induced crystal deposition in renal parenchyma and apoptotic changes in renal tubular epithelium into account, it may be useful to apply protective agents in an attempt to prevent or at least to limit the extent of the pathologic alterations. These agents should have properties both to prevent crystal deposition leading to renal destruction and to free oxygen radical formation. In the light of studies which have shown decreasing effects of losartan on oxidative stress [18] and on inflammatory cell regulation through NF-kappa B pathway [31], it is decided in this present study that application of losartan in the ethylene glycol group prevents the development of renal tissue damage and apoptosis by controlling RAS activation. These results can provide a new perspective in the treatment of some hyperoxaluric states by a reno-protective agent.

In conclusion, we showed that AT1 receptor blocker losartan can prevent tubulo-interstitial damage, which is believed to be caused by local AII and AT1 receptor response triggered by CaO_x crystal deposition in renal tissue, and tubular cell apoptosis, which is related to the increase of TGF-beta 1, bax and caspase-3 expressions.

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