Kidney International, Vol. 65 (2004), pp. 811–822

Tight blood pressure control decreases apoptosis during renal damage

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Tight blood pressure control decreases apoptosis during renal damage.

Background. An excess rate of apoptosis could lead to the gradual loss of renal mass. In this study, we investigated the role of apoptosis in the renal damage secondary to hypertension.

Methods. Spontaneously hypertensive rats with 5/6 renal mass reduction (subtotal nephrectomy) were distributed to receive no-treatment, 200 mg/L quinapril, 360 mg/L losartan, or triple therapy (200 mg/L hydralazine, 4 mg/L reserpine, and 100 mg/L hydrochlorothiazide) for 5 weeks. Sham-operated spontaneously hypertensive rats served as controls. Age-matched Wistar-Kyoto (WKY) rats, with or without subtotal nephrectomy, were also studied.

Results. Nontreated spontaneously hypertensive rats + subtotal nephrectomy developed proteinuria, glomerular sclerosis, and tubulointerstitial lesions. In comparison to spontaneously hypertensive rats, an increment in the number of [proliferating cell nuclear antigen (PCNA)]-positive and apoptotic [terminal deoxynucleotidyl transferase (Tdt)-mediated deoxyuridine triphosphate biotin nick end labeling (TUNEL)]-positive tubular and glomerular cells was observed. By contrast, WKY + subtotal nephrectomy rats showed less severe morphologic lesions, and only the number of proliferating cells increased. By Western blot, an up-regulation of renal Bax (apoptosis inducer) was noted both in spontaneously hypertensive rats + subtotal nephrectomy and WKY + subtotal nephrectomy rats. By contrast, Bcl-xL (apoptosis protector) was up-regulated in WKY + subtotal nephrectomy rats but not in spontaneously hypertensive rats + subtotal nephrectomy. The administration of appropriate doses of quinapril, losartan, or triple therapy to spontaneously hypertensive rats + subtotal nephrectomy normalized systolic blood pressure, partially prevented proteinuria, renal lesions and apoptosis, and decreased Bax, but no changes were noted in Bcl-xL. The Bax/Bcl-xL index was significantly increased in spontaneously hypertensive rats + subtotal nephrectomy compared to sham-operated spontaneously hypertensive rats and decreased in treated groups.

Conclusion. The combination of renal mass reduction and hypertension caused severe renal lesions associated to an increment of apoptosis rate, mainly in tubular epithelial cells. Tight blood pressure control decreased the apoptosis rate and morphologic lesions. These studies suggest that changes in the expression of apoptosis-regulatory genes contribute to the progressive damage in hypertensive rats with renal mass reduction.

Arterial hypertension is a leading cause of end-stage renal disease (ESRD); but also an important factor in the progression of other forms of chronic renal disease [1, 2]. Although the histologic manifestations of hypertensive nephrosclerosis are well known (glomerular and arterioarteriolar sclerosis associated with inflammatory cell infiltration, interstitial fibrosis, and tubular atrophy) [3], the pathophysiologic process that results in progressive renal failure secondary to hypertension remains unresolved.

Apoptosis, or programmed cell death, is a gene-regulated process that is now recognized to play an important role in maintaining cell number homeostasis both in health and disease (reviewed in [4, 5]). Cell deletion by apoptosis has been implicated in the repairing process of several renal diseases. In addition, unregulated excessive apoptosis can contribute to progressive chronic nephropathies, including hypertensive nephrosclerosis, by depletion of glomerular and tubular cells [4–6]. However, data on the response of renal cell apoptosis to adequate blood pressure control are scarce, and the molecular mechanisms are poorly understood.

Apoptosis is controlled in part by the Bcl-2 family of regulatory proteins (Bcl-2, Bcl-x, Bax, and others). Bcl-2 can prevent or delay many forms of apoptosis [7]. Bcl-x has two alternatively spliced forms: Bcl-xL that protects

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Key words: apoptosis, experimental hypertension, renal mass reduction, renin-angiotensin system.

Received for publication November 19, 2002
and in revised form June 30, 2003, and September 8, 2003
Accepted for publication October 8, 2003

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cells from a wide variety of apoptotic stimuli; and Bcl-xS that has opposing effects to Bcl-2 and Bcl-xL [5, 7]. Bax binds to and antagonizes the protective effect of Bcl-2 and Bcl-xL [8]. In this sense, the ratio of expression of Bcl-2 or Bcl-xL to Bax or Bcl-xS appears to determine cell susceptibility to apoptosis when the microenvironment is adverse for survival. In adult organs, including the kidney, the levels of Bcl-xL appear higher than those of Bcl-2 [9].

The present study was designed to investigate the role of apoptosis in the progressive renal damage associated to hypertension and whether the expression of apoptosis-related genes was altered in these animals. In order to assess this hypothesis, we have employed spontaneously hypertensive rats, an experimental model considered to be similar in many aspects to hypertension in humans. Spontaneously hypertensive rats with intact renal mass have normal renal autoregulation, and develop systemic hypertension and vascular and renal injury over the years. However, subtotal nephrectomy leads to an increment in the glomerular capillary pressure and accelerates glomerulosclerosis in a pattern similar to that observed in malignant hypertension [10]. In addition, we have studied apoptosis in response to the tight blood pressure control with three antihypertensive therapy regimes [i.e., angiotensin-converting enzyme (ACE) inhibition, angiotensin II receptor antagonism, and triple therapy].

METHODS

Experimental model

Studies were performed in 8-week-old male spontaneously hypertensive rats (Criffa, Barcelona, Spain). All rats had free access to standard rat chow and water. Animals underwent subtotal nephrectomy as a two-step procedure: ligation of two or three branches of the left renal artery, followed, 7 days later, by total right nephrectomy. Just after the right nephrectomy, spontaneously hypertensive rats were randomly distributed into different groups: (*1*) nontreated group (spontaneously hypertensive rats + subtotal nephrectomy), animals with spontaneous development of the disease ($N = 7$); (*2*) quinapril group (quinapril + spontaneously hypertensive rats + subtotal nephrectomy), animals that received 200 mg/L quinapril (as powdered hydrochloride salt) (Pfizer, Madrid, Spain) in the drinking water ($N = 7$); (*3*) losartan group (losartan + spontaneously hypertensive rats + subtotal nephrectomy), animals receiving 360 mg/L losartan (Merck Sharp & Dohme, Madrid, Spain) in the drinking water ($N = 8$); and (*4*) triple-therapy group (triple therapy + spontaneously hypertensive rats + subtotal nephrectomy), animals receiving 200 mg/L hydralazine, 4 mg/L reserpine, and 100 mg/L hydrochlorothiazide in the drinking water ($N = 5$).

A pilot study of 2-week duration was performed in order to obtain the dose of losartan, quinapril, and triple therapy (hydralazine, reserpine, and hydrochlorothiazide) that decreases and maintains systolic blood pressure within normal range.

At the same time, a group of spontaneously hypertensive rats underwent a sham operation ($N = 6$) and served as controls. Two further groups of age-matched normotensive Wistar-Kyoto (WKY) rats, with or without subtotal nephrectomy ($N = 4$ and 5, respectively), were also studied.

After 5 weeks of study, at 14 weeks of age, all rats were anesthetized with pentobarbital sodium (5 mg/100 g body weight) and kidneys were perfused with cold sodium saline and removed.

Measurement of systolic blood pressure and renal function

Systolic blood pressure was measured weekly in conscious animals by a tail-cuff sphygmomanometer (NARCO Biosystems, Austin, TX, USA). Periodically, animals were maintained in metabolic boxes during 24 hours in order to collect the urine. Total urinary protein excretion was quantified by the method of the sulfosalicylic acid [11]. Renal function (measured as creatinine clearance) was calculated from a urine sample taken 24 hours before the animal sacrifice.

Renal histopathologic studies

For light microscopy, paraffin-embedded renal sections (4 μm thick) were prepared and stained with hematoxylin-eosin and Masson's trichrome. For each animal, the percentage of glomeruli exhibiting global or segmental sclerosis was determined. Sclerosis was evidenced by an increase in mesangial matrix and/or collapse and condensation of the glomerular basement membrane [12]. Tubulointerstitial injury (defined as tubular dilation and/or atrophy, interstitial fibrosis and inflammatory cell infiltrate) were graded by the following semiquantitative score [13]: 0, no changes; 1, focal changes that involve 25% of the sample; 2, changes affecting >25% to 50% of the sample; 3, changes involving >50% to 75%; 4, lesions affecting >75% of the sample. In all cases, renal biopsies were only taken from the center of the noninfarcted area. All these studies were performed in a blinded fashion by two observers.

Immunohistochemistry

Immunolocalization of proliferating cell nuclear antigen (PCNA) and apoptosis-related proteins Bax and Bcl-xL was performed in paraffin-embedded renal tissues. Briefly, 4 μm thick sections were dewaxed and rehydrated by descending ethanol concentrations. For PCNA detection, sections were also treated by oven heating in 0.01 mol/L sodium citrate, pH 6.0, for 10 minutes.

After the quenching of endogenous peroxidase activity, sections were incubated with a prediluted monoclonal mouse antirat PCNA (PC-10) (Zymed Laboratories, Inc., San Francisco, CA, USA), a rabbit polyclonal antimouse/rat Bax antibody (BD Bioscience, Madrid, Spain) diluted 7 μL/mL or a rabbit polyclonal antihuman Bcl-xS/L antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA.) diluted 1:50 overnight at 4°C in a humid atmosphere. Thereafter, sections were processed using avidin-biotin immunoperoxidase method [AB-Complex/horseradish peroxidase (HRP)] (Dako A/S, Glostrup, Denmark) with 3-3′-diamino-benzidine (DAB) (Sigma, Madrid, Spain) as the chromogen. The tissue sections were counterstained with Mayer's hematoxylin (Sigma). Negative controls were performed in parallel for specific labelings by replacing the primary antibody with a nonimmune normal rabbit serum. PCNA-positive cells were quantified by image analysis as described below. Bax and Bcl-xS/L immunostaining results were evaluated by two independent observers in a blinded fashion.

In situ detection of apoptosis

Apoptosis was detected by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labeling (TUNEL) using a commercial kit (FragEl^{TM} DNA Fragmentation Detection Kit) (Oncogene Research Products, Cambridge, MA, USA). Briefly, after deparaffinization and rehydration, renal sections (4 μm thick) were treated with 20 μg/mL proteinase K and immersed in 3% H_2O_2 in methanol for 10 minutes. Slides were rinsed with Tris-buffered saline (TBS), immersed in TdT equilibration buffer, and then incubated with TdT and biotinylated-dUTP at 37°C for 2 hours. For negative controls, sections were incubated in TdT buffer without TdT. The reactions were stopped with 0.5 mol/L ethylenediaminetetraacetic acid (EDTA), pH 8. Biotinylated-dUTP-labeled DNA was detected and visualized using a streptavidin-HRP conjugated and DAB (Sigma) as the chromogen. Tissue sections were counterstained with Mayer's hematoxylin (Sigma) or methyl green.

Counts of proliferating (PCNA-positive) and apoptotic (TUNEL-positive) cells

The number of cells positive to PCNA and TUNEL were quantified in a blinded fashion with computer-assisted image analysis software (Optimas 6.5) (Media Cybernetics, Silver Spring, MD, USA) and digitized images. For each renal cortex section, proliferating glomerular cells were calculated by counting at least 20 glomeruli per biopsy and expressed as the mean number ± SEM per glomeruli. In tubulointerstitium, proliferating cells were calculated by counting all positive PCNA cells in four to six not overlapping random fields viewed at ×200 magnification (excluding glomeruli) and expressed as the mean number ± SEM per field. Only intense brown/black-stained nuclei were considered as PCNA-positive staining. The corresponding number of cells undergoing apoptosis was calculated in the same manner. Only intense brown/black-stained nuclei, including pyknotic nuclei with apoptotic bodies that stained positive, were considered as TUNEL-positive staining.

Western blot

Tissue samples were homogenized in lysis buffer [50 mmol/L TrisHCl, 150 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L ethylene glycol tetraacetate (EGTA), 0.2% Triton X-100, 0.3% NP-40, 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 1 μg/mL pepstatin A] and then separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. After electrophoresis, samples were transferred to polyvinyldene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA, USA), blocked with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)/0.5% Tween-20 (vol/vol) for 1 hour, washed with PBS/Tween-20, and incubated with the following antibodies: rabbit polyclonal antimouse Bax antibody (1:500) (Santa Cruz Biotechnology, Inc.) and rabbit polyclonal antihuman Bcl-xS/L (1:500) (Santa Cruz Biotechnology, Inc.). Although the anti-Bcl-x antibody employed recognized both S and L forms, only a unique band corresponding to the L form was observed in all groups of rats (Fig. 5). Blots were washed with PBS/Tween-20 and subsequently incubated with HRP-conjugated antirabbit IgG (1:2000) (Amersham, Aylesbury, UK). After washing with PBS/Tween-20 the blots were developed with the chemiluminescence method (ECL) (Amersham).

Statistical analysis

Results are expressed as mean ± SEM. Data from multiple groups were compared using the unpaired Student *t* test or the Kruskal-Wallis nonparametric analysis of variance (ANOVA) test when appropriate. Differences were considered significant if the P value was less than 0.05.

RESULTS

Evolution of the systolic blood pressure and proteinuria

Severe hypertension was already present in 8-week-old spontaneously hypertensive rats at the beginning of the study (187 ± 3 vs. WKY, 108 ± 6 mm Hg) ($N = 10$ to 28 per group, $P < 0.05$). Immediately after the right nephrectomy, spontaneously hypertensive rats were randomized into five groups: (*1*) spontaneously hypertensive

Table 1. Proteinuria in normotensive Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) with and without subtotal nephrectomy (SNx) at baseline and at end of the follow-up period. Some SHR with SNx received quinapril (Q) (200 mg/L), losartan (L) (360 mg/L), or triple therapy (TT) (200 mg/L hydralazine, 4 mg/L reserpine, 100 mg/L hydrochlorothiazide) in the drinking water for 5 weeks

	Proteinuria *mg/24 hours*	
	Basal values	After 5 weeks of study
WKY ($N = 4$)	9 ± 2	8 ± 1
WKY + SNx ($N = 5$)	9 ± 1	$13 \pm 2^{a,c}$
SHR ($N = 6$)	15 ± 2	21 ± 1^{a}
SHR + SNx ($N = 7$)	18 ± 1	$47 \pm 7^{a,b}$
Q + SHR + SNx ($N = 7$)	20 ± 1	$24 \pm 4^{a,c}$
L + SHR + SNx ($N = 8$)	25 ± 3	$23 \pm 1^{a,c}$
TT + SHR + SNx ($N = 5$)	18 ± 1	$22 \pm 3^{a,c}$

Data represent mean ± SEM.
[a] $P < 0.05$ vs. WKY at the same age.
[b] $P < 0.05$ vs. SHR at the same age.
[c] $P < 0.05$ vs. SHR + SNx at the same age.

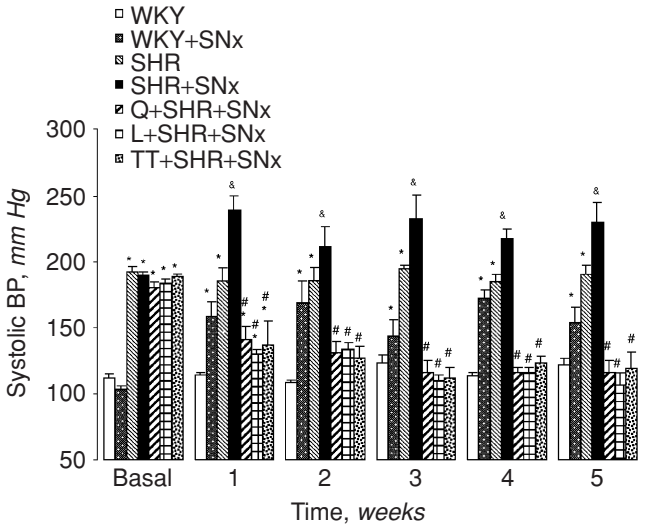


Fig. 1. Serial values of systolic blood pressure during the 5-week period of study in normal Wistar-Kyoto (WKY) rats, WKY with subtotal nephrectomy (SNx), spontaneously hypertensive rats (SHR), SHR + SNx, quinapril(Q)-treated SHR + SNx, losartan (L)-treated SHR + SNx, and triple therapy(TT)-treated SHR + SNx. Data represent mean ± SEM, $N = 4$ to 8 animals per group. $^{*}P < 0.05$ with respect to WKY; $\#P < 0.05$ with respect to SHR + SNx without treatment.

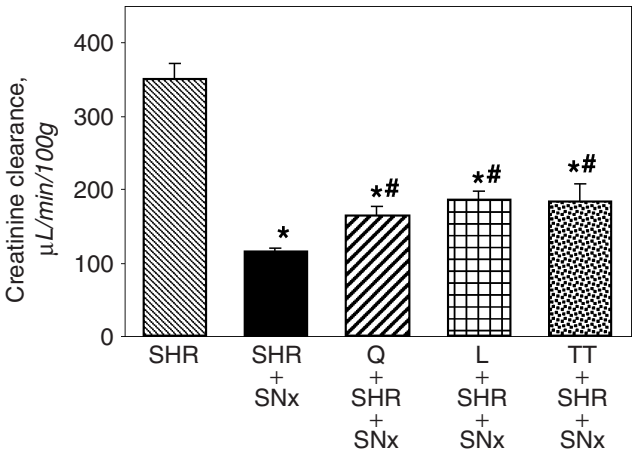


Fig. 2. Renal function at the end of the 5-week period of study in spontaneously hypertensive rats (SHR), SHR + subtotal nephrectomy (SNx), quinapril (Q)-treated SHR + SNx, losartan (L)-treated SHR + SNx, and triple therapy (TT)-treated SHR + SNx. Data represent mean ± SEM, $N = 5$ to 8 animals per group. $^{*}P < 0.01$ with respect to SHR + SNx; $\#P < 0.01$ with respect to SHR + SNx without treatment.

rats + subtotal nephrectomy; (*2*) quinapril + spontaneously hypertensive rats + subtotal nephrectomy; (*3*) losartan + spontaneously hypertensive rats + subtotal nephrectomy; (*4*) triple therapy + spontaneously hypertensive rats + subtotal nephrectomy; and (*5*) sham-operated spontaneously hypertensive rats animals. At this time, neither the proteinuria nor the systolic blood pressure differed between spontaneously hypertensive rats from the different groups (Table 1) (Fig. 1). By contrast, at the end of the study, spontaneously hypertensive rats + subtotal nephrectomy showed significantly higher urinary protein excretion and systolic blood pressure than sham-operated spontaneously hypertensive rats (Table 1) (Fig. 1). The reduction of renal mass in WKY rats also increased proteinuria and systolic blood pressure, although without reaching spontaneously hypertensive rats values (Table 1) (Fig. 1).

The administration of quinapril (200 mg/L), losartan (360 mg/L), and triple therapy (200 mg/L hydralazine, 4 mg/L reserpine, 100 mg/L hydrochlorothiazide) to spontaneously hypertensive rats + subtotal nephrectomy decreased significantly systolic blood pressure after 1 week of treatment (Fig. 1). After 2 weeks, all treatments adequately controlled systolic blood pressure, which remained at normotensive range throughout the rest of the study period (Fig. 1). Treatment with quinapril, losartan, or triple therapy prevented the appearance of severe proteinuria (table 1), and resulted in significantly preserved renal function (Fig. 2).

Study of the histologic lesions

At the end of the study, normotensive sham-operated animals did not show evidence of renal lesion. Hypertensive sham-operated animals showed occasional sclerotic glomeruli and some inflammatory cells. Both in normotensive and hypertensive animals, subtotal nephrectomy induced a significant increment in the number of sclerotic glomeruli, as well as marked tubulointerstitial damage (tubular atrophy, interstitial inflammatory cells, and fibrosis) with respect to sham-operated control groups (Fig. 3). Morphologic lesions displayed by spontaneously hypertensive rats + subtotal nephrectomy were more severe than those in WKY + subtotal nephrectomy.

The administration of quinapril, losartan, or triple therapy to spontaneously hypertensive rats + subtotal

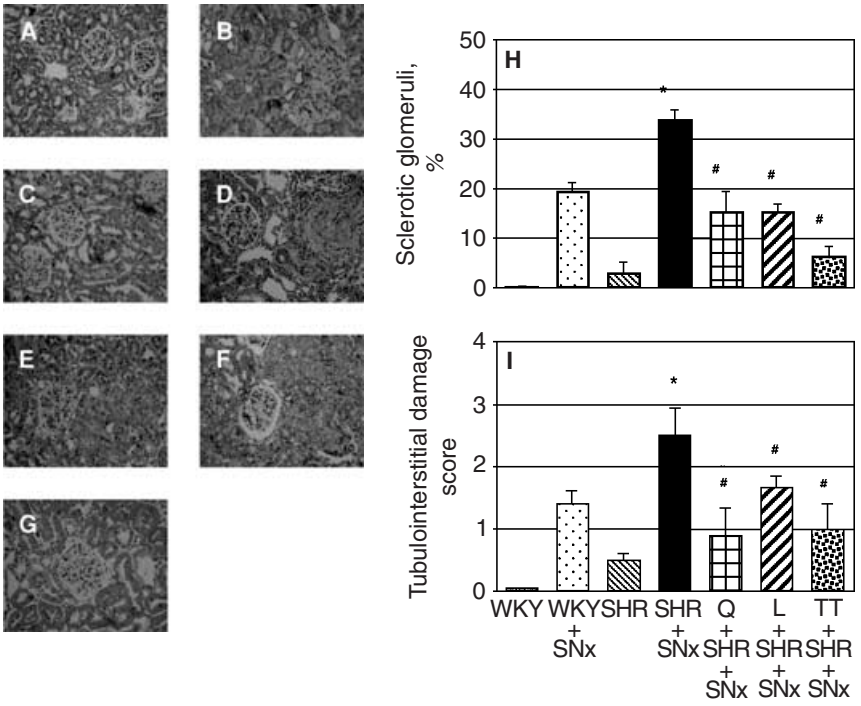


Fig. 3. Renal tissue from normal Wistar-Kyoto (WKY) rats (*A*), WKY + subtotal nephrectomy (SNx) (*B*), spontaneously hypertensive rats (SHR) (*C*), SHR + SNx (*D*), quinapril (Q)-treated SHR + SNx (*E*), losartan (L)-treated SHR + SNx (*F*), and triple therapy (TT)-treated SHR + SNx (*G*). SHR (C) did not show important renal morphologic lesions. By contrast, WKY + SNx (B) and SHR + SNx (D) presented marked glomerular and tubulointerstitial damage, being the lesions more severe in SHR + SNx than in WKY + SNx. The administration of quinapril, losartan, or triple therapy to SHR + SNx improved significantly renal damage (E, F and G, respectively), compared to SHR + SNx without treatment (magnification ×10). Percentage (%) of sclerotic glomeruli (*H*) and histologic tubulointerstitial damage score (*I*) of all studied groups. Data represent mean ± SEM, $N = 4$ to 8 animals per group. $^{*}P < 0.05$ with respect to WKY + SNx; $\#P < 0.05$ with respect to SHR + SNx without treatment.

nephrectomy ameliorated renal damage, although morphologic lesions were not completely normalized in any group (Fig. 3).

Localization of cell proliferation in renal tissue

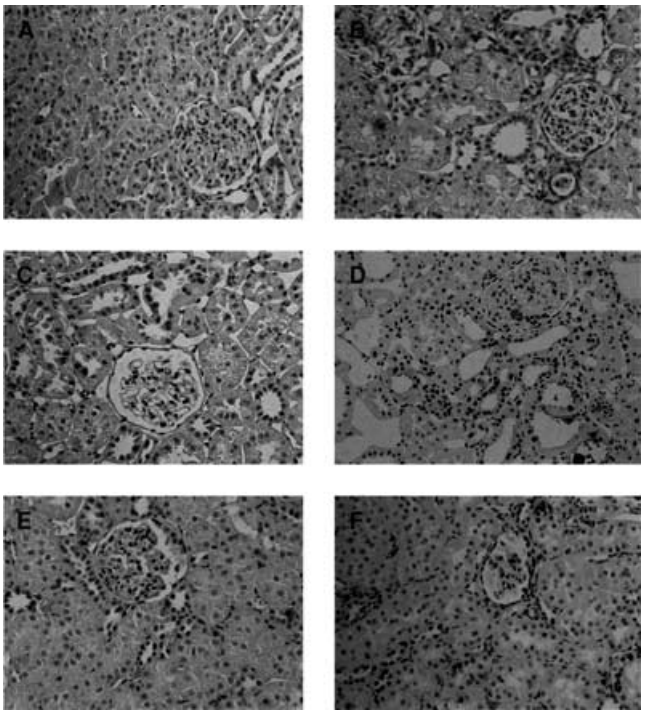
At the end of the study, sham-operated spontaneously hypertensive rats showed higher number of PCNA-positive cells, mainly in the interstitial and tubular compartments, than WKY rats (Fig. 4), coinciding with previous studies that demonstrate that cells from genetically hypertensive rats proliferate at a higher rate than those of normotensive origin [14]. Both spontaneously hypertensive rats and WKY with subtotal nephrectomy showed an increment in the number of glomerular, tubular, and interstitial proliferating cells with respect to sham-operated controls (Fig. 4). By light microscopy, mitotic figures were also observed within the tubular and glomerular cells of spontaneously hypertensive rats + subtotal nephrectomy (Fig. 5). Spontaneously hypertensive rats + subtotal nephrectomy receiving quinapril, losartan, or triple therapy showed less proliferating glomerular, tubular, and infiltrating cells compared to spontaneously hypertensive rats + subtotal nephrectomy without treatment (Fig. 4).

Localization of apoptotic cells in renal tissue

Figure 6 shows photomicrographs of TUNEL immunohistochemistry of renal cortex from WKY and spontaneously hypertensive rats with and without subtotal nephrectomy. Only very few cells were identified as apoptotic in sham-operated WKY rats, spontaneously hypertensive rats and WKY rats + subtotal nephrectomy. However, in spontaneously hypertensive rats + subtotal nephrectomy many cells showing an intense brown TUNEL-stained nuclei were observed in tubules (detail in Fig. 7), interstitium, and vessels. In glomeruli, apoptotic cells were seen, including epithelial cells from Bowman's capsule and podocytes (Figs. 6 and 8). The administration of antihypertensive therapies to spontaneously hypertensive rats + subtotal nephrectomy decreased significantly the number of apoptotic cells, both in glomeruli and tubulointerstitium, in comparison to nontreated spontaneously hypertensive rats + subtotal nephrectomy (Fig. 6). Table 2 shows the localization of apoptotic cells in the different groups. Interestingly, podocyte apoptosis was not detected in spontaneously hypertensive rats + subtotal nephrectomy treated with quinapril, losartan, or triple therapy. These observations suggest that loss of podocytes by apoptosis could be an important event in the progression of glomerulosclerosis. Detailed studies are ongoing in our laboratory to determine the molecular mechanisms implicated in this process.

Expression of apoptosis-related proteins

In comparison with sham-operated rats, both WKY + subtotal nephrectomy and spontaneously hypertensive rats + subtotal nephrectomy showed a significant increment in Bax levels in the renal cortex (Fig. 9A). However, increased expression of Bcl-xL was noted in WKY rats + subtotal nephrectomy with respect to WKY rats, but it



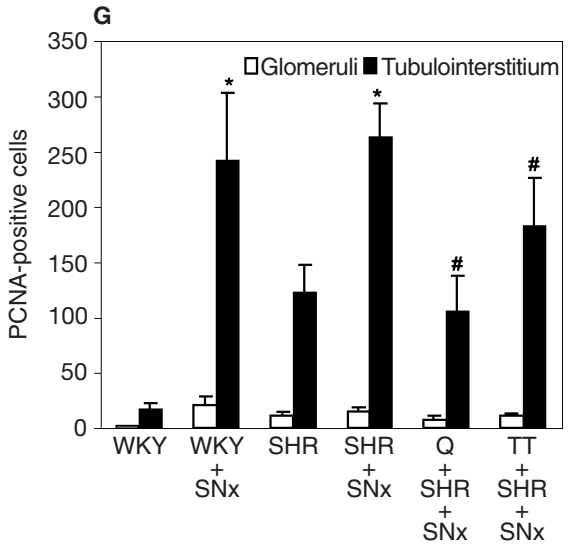


Fig. 4. Localization of cell proliferation by proliferating cell nuclear antigen (PCNA) immunolocalization in renal tissue from normal Wistar-Kyoto (WKY) rats (*A*), WKY + subtotal nephrectomy (SNx) (*B*), spontaneously hypertensive rats (SHR) (*C*), SHR + SNx (*D*), quinapril (Q)-treated SHR + SNx (*E*), and triple therapy (TT)-treated SHR + SNx (*F*). Many cells positive for PCNA were identified in glomeruli and tubulointerstitium from WKY + SNx and SHR + SNx. The administration of quinapril or triple therapy to SHR + SNx decreased significantly the number of proliferative cells in comparison with nontreated SHR + SNx (magnification ×10). (*G*) Quantification of PCNA-positive cells by image analysis. Data represent mean ± SEM, $N = 4$ to 8 animals per group. $^{*}P < 0.05$ with respect to the same strain without SNx; $^{\#}P < 0.05$ with respect to SHR + SNx without treatment.

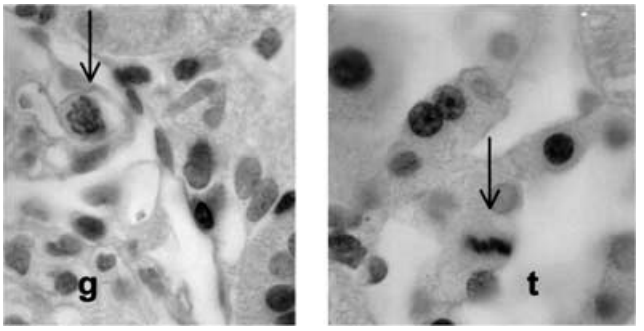


Fig. 5. Mitotic cells (arrows) in glomeruli (g) and tubule (t) from a representative spontaneously hypertensive rat + subtotal nephrectomy (SHR + SNx). Some mitotic motifs showing signs of chromosome separation and chiasmata formation were detected in SHR + SNx (magnification ×40).

was not significantly modified in spontaneously hypertensive rats + subtotal nephrectomy compared to spontaneously hypertensive rats (Fig. 9B).

The administration of losartan, quinapril, or triple therapy to spontaneously hypertensive rats + subtotal nephrectomy reduced significantly Bax expression with respect to spontaneously hypertensive rats + subtotal nephrectomy without treatment (Fig. 9A). By contrast, animals receiving losartan, quinapril, or triple therapy did not show a significant modification in the Bcl-xL expression in renal cortex with respect to nontreated hypertensive rats (Fig. 9B). In spontaneously hypertensive rats + subtotal nephrectomy, these changes resulted in an increased Bax/Bcl-xL ratio, an index of the susceptibility of the cells to apoptosis. The Bax/Bcl-xL ratio was decreased by the administration of the antihypertensive treatments (Fig. 10).

Immunohistochemistry

Localization of Bax and Bcl-xL proteins is shown in Figures 11 and 12. Both Bax and Bcl-xL immunostaining was predominantly confined in proximal and distal tubules and collecting ducts, and to a lesser extent in glomeruli. In WKY rats and spontaneously hypertensive rats without subtotal nephrectomy no positive immunostaining was detected. Confirming findings obtained with Western blot, no differences were observed in Bax staining between WKY rats + subtotal nephrectomy and spontaneously hypertensive rats + subtotal nephrectomy animals (Fig. 11); however, Bcl-xL immunostaining was decreased in spontaneously hypertensive rats + subtotal nephrectomy in comparison with WKY rats + subtotal nephrectomy rats (Fig. 12). Negative controls of the immunohistochemistry showing the specificity of the Bax and Bcl-xL antibodies are shown in Figures11H and 12H, respectively.

Semiquantification of Bax and Bcl-xL immunostaining is shown in Table 3.

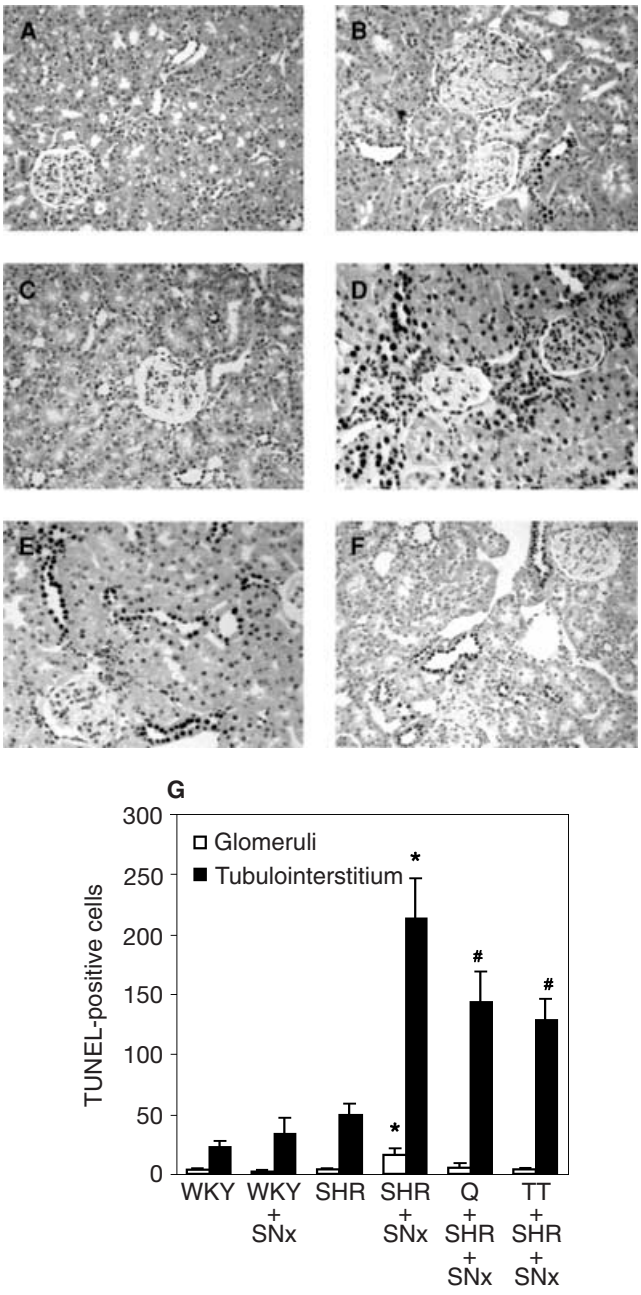


Fig. 6. Localization of apoptotic cells in renal tissue from normal Wistar-Kyoto (WKY) rats (*A*), WKY + subtotal nephrectomy (SNx) (*B*), spontaneously hypertensive rats (SHR) (*C*), SHR + SNx (*D*), quinapril (Q)-treated SHR + SNx (*E*), and triple therapy (TT)-treated SHR + SNx (*F*). Few apoptotic cells were observed in glomeruli or tubulointerstitium in WKY, WKY + SNx, and SHR. By contrast, many apoptotic cells were observed in SHR + SNx. The administration of quinapril or triple therapy to SHR + SNx rats decreased significantly the number of apoptotic cells in comparison with nontreated SHR + SNx (magnification ×10). (*G*) Quantification of terminal deoxynucleotidyl transferase (Tdt)-mediated deoxyuridine triphosphate biotin nick end labeling (TUNEL)-positive cells by image analysis. Data represent mean ± SEM, $N = 4$ to 8 animals per group. $^*P < 0.05$ with respect to SHR; $\#P < 0.05$ with respect to SHR + SNx without treatment.

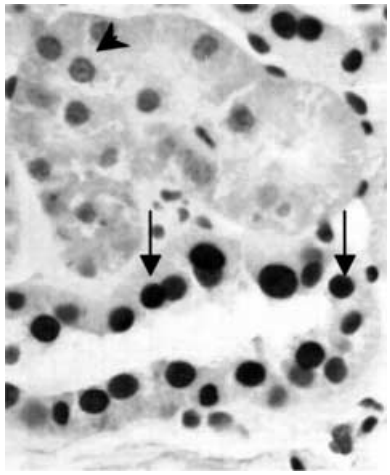


Fig. 7. Detail of tubular nuclei that show intense brown terminal deoxynucleotidyl transferase (Tdt)-mediated deoxyuridine triphosphate biotin nick end labeling (TUNEL) staining (arrows), in contrast to the surrounding normal tubular nuclei (arrowhead), of a representative spontaneously hypertensive rat + subtotal nephrectomy (SHR+SNx) rat (magnification ×40).

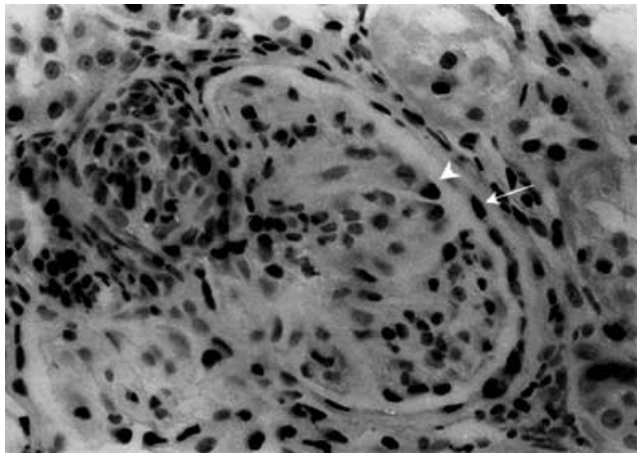


Fig. 8. Detail of apoptotic cells in glomeruli from a representative spontanteously hypertensive +subtotal nephrectomy (SHR + SNx) rat. Some epithelial cells from Bowman's capsule (arrows) and podocytes (arrowhead) showed and intense terminal deoxynucleotidyl transferase (Tdt)-mediated deoxyuridine triphosphate biotin nick end labeling (TUNEL)-stained nuclei (magnification ×40).

DISCUSSION

Our results show that renal mass reduction led to more severe renal lesions in spontaneously hypertensive rats than in WKY rats and, indeed, to an increment in the number of renal apoptotic cells in spontaneously hypertensive rats + subtotal nephrectomy but not in WKY rats + subtotal nephrectomy. Tight control of blood pressure with either an ACE inhibitor, an angiotensin II type 1 receptor (AT1) antagonist or triple-therapy was associated with decreased proteinuria, renal lesions, cell turnover and apoptosis rate.

Table 2. Localization of terminal deoxynucleotidyl transferase (Tdt)-mediated deoxyuridine triphosphate biotin nick end labeling (TUNEL)-positive cells in normotensive Wistar-Kyoto (WKY) rats, spontaneously hypertensive rats (SHR) with and without subtotal nephrectomy (STNx). Some SHR with STNx received quinapril (Q) (200 mg/L) or triple therapy (200 mg/L hydralazine, 4 mg/L reserpine, 100 mg/L hydrochlorothiazide) during 5 weeks

	Glomeruli	Tubular cells	Vascular cells	Interstitial cells
WKY	–	–	–	–
WKY +STNx	–	–/+	–	–/+
SHR	–	–/+	–	–/+
SHR + STNx	+	+++	++	++
Q + SHR + STNx	–	+	+	–
TT + SHR + STNx	–	+	+	+

Nuclear DNA fragmentation was detected by TUNEL technique as described in the **Methods** section. Data represent four to eight rats per group.

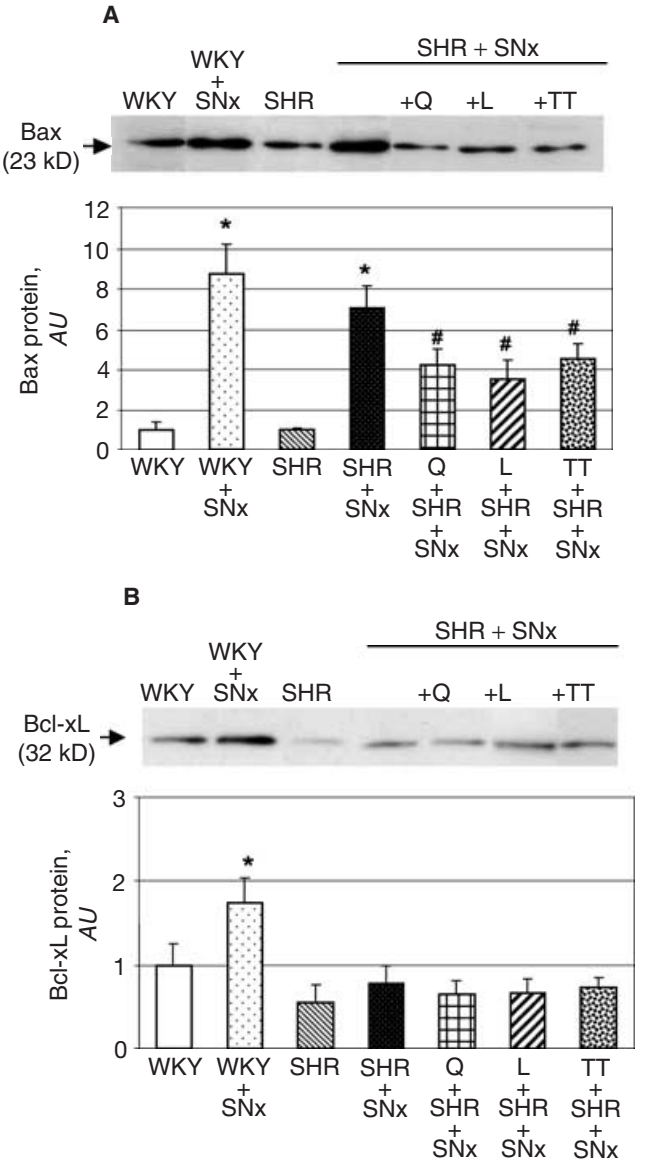


Fig. 9. Bax (*A*) and Bcl-xL (*B*) protein expression in the renal cortex from normal Wistar-Kyoto (WKY) rats, WKY + subtotal nephrectomy (SNx), spontaneously hypertensive rats (SHR), SHR + SNx, quinapril (Q)-treated SHR + SNx, losartan (L)-treated SHR + SNx, and triple therapy (TT)-treated SHR + SNx. Upper panels, Western blot results showing a representative animal from each group. Four to eight animals per group were analyzed. Lower panels, Western blot densitometric results. Results are expressed as the fold-increase vs. sham-operated WKY rats. Data represent mean ± SEM. $^*P < 0.05$ vs. the same strain without SNx; $\#P < 0.05$ vs. SHR + SNx without treatment. AU is arbitrary units.

Early stages of renal injury involve compensatory renal growth associated with hypertrophy and hyperplasia [15]. By contrast, the late renal fibrotic stage is characterized by the progressive loss of glomerular and tubular cells, and their replacement by fibrous tissue, leading to chronic tubulointerstitial injury and glomerular sclerosis [16]. In general, proliferative and apoptotic changes take place simultaneously, as a counterbalancing homeostatic mechanism of cell number. It is well known that after infarction of 5/6 of the kidneys an increment in the proliferative processes and cellular hyperplasia is observed as an adaptative response to the hemodynamic changes [17]. In our study, increased cell proliferation, not balanced by increased apoptosis, was observed in WKY rats + subtotal nephrectomy. By contrast, increased cell turnover (both increased proliferation and increased apoptosis) was noted in spontaneously hypertensive rats with subtotal nephrectomy. It is known that the time necessary for a complete proliferative cell cycle is around fivefold longer than the apoptotic event [18, 19]. In addition Thomas et al [20] demonstrated that some cells expressing PCNA also stained positive by TUNEL, suggesting that the expression of PCNA may be a common cellular event in proliferation and apoptosis. Therefore, in our study the numerical match of the proliferative and apoptotic changes would represent a predominance of the apoptotic ones in spontaneously hypertensive rats with subtotal nephrectomy, contributing to the loss of renal parenchymal cells. Since both normotensive and hypertensive animals have the same reduction of renal mass and similar changes in renal cell proliferation, hypertension, or the genetic background could contribute to the excess apoptosis observed in spontaneously hypertensive rats + subtotal nephrectomy. In this regard, in target organs of hypertension, mainly heart, kidney and brain, apoptosis paralleling hypertrophy/hyperplasia has been demonstrated, although a dissociation between both processes has also been observed [21]. Hamet et al [21] described an apoptotic window in the hearts of spontaneously hypertensive rats only during the development (8 to 16 weeks) of hypertension that fell below the levels observed in normotensive animals during established hypertension (24 weeks of age).

We examined the effect of different antihypertensive therapies on apoptosis in spontaneously hypertensive rats + subtotal nephrectomy. The administration of the ACE

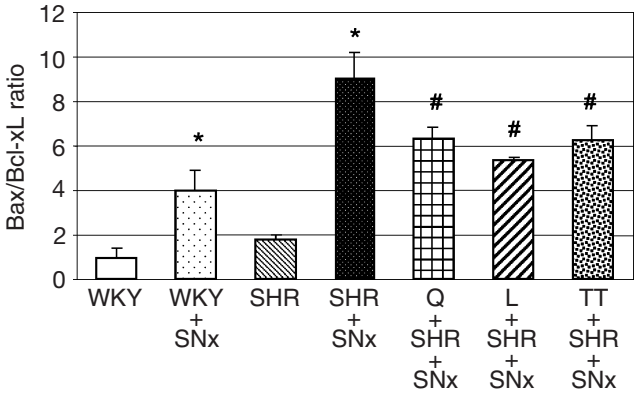


Fig. 10. Bax/Bcl-xL ratio in normal Wistar-Kyoto (WKY) rats, WKY +subtotal nephrectomy (SNx), spontaneously hypertensive rats (SHR), SHR + SNx, quinapril (Q)-treated SHR + SNx, losartan (L)-treated SHR + SNx, and triple therapy (TT)-treated SHR + SNx. Data represent mean ± SEM, $N = 4$ to 8 animals per group. $^{*}P < 0.05$ respect to the same strain without SNx; $\#P < 0.05$ vs. SHR + SNx without treatment.

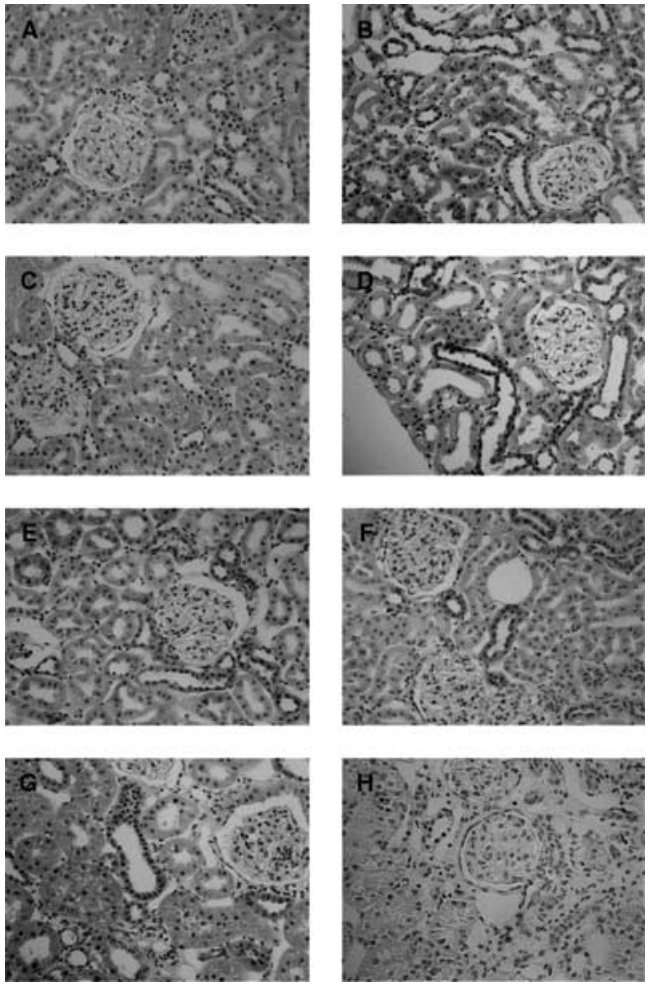


Fig. 11. Immunolocalization of Bax in the kidney from normal Wistar-Kyoto (WKY) rats (*A*), WKY + subtotal nephrectomy (SNx) (*B*), spontaneously hypertensive rats (SHR) (*C*), SHR + SNx (*D*), quinapril (Q)-treated SHR + SNx (*E*), losartan (L)-treated SHR + SNx (*F*), and triple therapy (TT)-treated SHR + SNx (*G*). A marked increase in the intensity of the staining in proximal and distal tubules was observed in WKY + SNx and SHR + SNx. (*H*) Renal sections incubated with a non-immune normal rabbit serum instead of Bax antibody demonstrates no immunostaining (magnification ×20).

inhibitor quinapril, the AT1 antagonist, losartan, or triple therapy to spontaneously hypertensive rats with subtotal nephrectomy for 5 weeks normalized systolic blood pressure, and reduced proteinuria, renal lesions and the number of apoptotic cells. It is well known that the renin-angiotensin system is activated in the remnant kidney nephropathy[22]. Angiotensin II (Ang II), the main peptide of the renin-angiotensin system, can act as a growth factor, inducing, via a nonhemodynamic effect, cell proliferation, hypertrophy, and expression of growth factors, through the activation of its AT1 receptors [23, 24]. Therefore, reduced apoptosis in response to antihypertensive therapies may be, at least partially, a consequence of reduced cellular proliferation. However, the role of the renin-angiotensin system in apoptosis has been demonstrated more directly by the finding that Ang II administration can induce apoptosis both in vivo and in vitro [25–27]. Interestingly, Ang II induces apoptosis depending on the cell type and which receptor subtype is bound. In vitro, it has been shown that the AT2 receptor exerts apoptotic effects in different cells types, including tubular epithelial cells [28, 29]. In vivo, we have recently demonstrated that increased AT2 receptor-induced apoptosis could be implicated in the renal lesions displayed by rats with persistent proteinuria. However, AT1 receptors play a more important proapoptotic role in cardiovascular cell types, such as cardiomyocytes and vascular smooth muscle cells [25, 30, 31]. More recently, it has been demonstrated that Ang II induces an exaggerated apoptotic response in cardiomyocytes from adult WKY rats [32]. Furthermore, whereas Ang II-induced apoptosis was prevented by AT1 blockade in cardiomyocytes from adult WKY normotensive rats, blockade of both the AT1 and the AT2 receptor was needed to blunt the apoptotic response to Ang II in SHR cardiomyocytes [32]. It has been proposed that Ang II-induced apoptosis depends on the ratio between the expression of AT1 and AT2 receptors. In this sense, we have observed that spontaneously hypertensive rats with subtotal nephrectomy showed an increment in the AT2 gene expression coinciding with the enhanced number of apoptotic cells (unpublished data), suggesting a potential mechanism by which Ang II could induce apoptosis. Recently Miura and Karnik [33] have proposed that AT2 receptor overexpression is a signal for apoptosis, independently of Ang II binding. Further studies are needed in order to assess the role of the Ang II receptors in our model of subtotal nephrectomy.

A somewhat surprising result was the fact that, in our model, triple therapy decreased PCNA- and TUNEL-positive cells and Bax protein to a similar extent to that

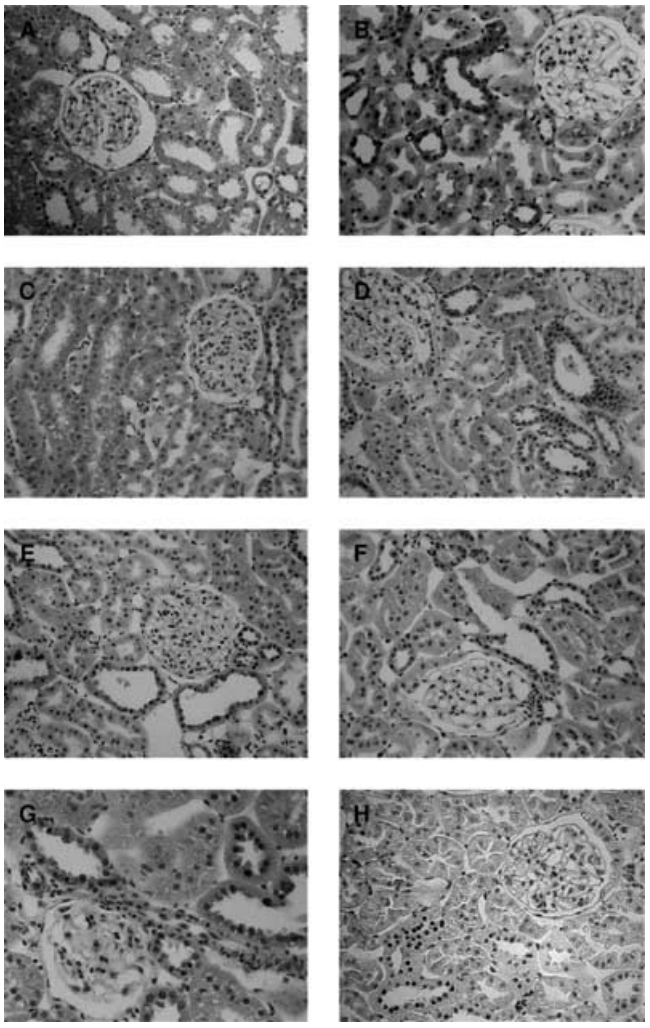


Fig. 12. Immunolocalization of Bcl-xL in the kidney from normal Wistar-Kyoto (WKY) rats (*A*), WKY + subtotal nephrectomy (SNx) (B), spontaneously hypertensive rats (SHR) (*C*), SHR + SNx (*D*), quinapril (Q)-treated SHR + SNx (*E*), losartan (L)-treated SHR + SNx (*F*), and triple therapy (TT)-treated SHR + SNx (*G*). A marked increase in the intensity of the staining in proximal and distal tubules was observed only in WKY + SNx. (*H*) Renal sections incubated with a non-immune normal rabbit serum instead of Bcl-xS/L antibody demonstrates no immunostaining (magnification ×20).

Table 3. Distribution of Bax and Bcl-xL staining is summarized

	Bax		Bcl-xL	
	Glomeruli	Tubules	Glomeruli	Tubules
WKY	–	–	–	–
WKY + STNx	–	+++	–/+	+++
SHR	–	–/+	–	–
SHR + STNx	–/+	+++	–	+
Q + SHR + STNx	–/+	++	–	+
L + SHR + STNx	–/+	++	–	+
TT + SHR + STNx	–/+	++	–/+	+

Data represent four to eight rats per group. Intensity was quantified as follows: +, low; ++, medium; +++, high; –, absence of signal.

of quinapril and losartan, suggesting that the beneficial effect of therapy depends on blood pressure control, at least in the early stages of renal injury. Indeed, a similar beneficial effect of triple therapy on this model had been previously noted, although the influence on apoptosis was not studied[10]. Tea et al [34] have reported that apoptosis is regulated in a time- and organ-specific manner by cardiovascular drugs in vivo. Because we have investigated only one single time point, the effects of losartan, quinapril, and triple therapy on renal apoptosis with an earlier or more prolonged schedule of drug administration is unknown. In addition, an indirect effect of triple therapy on renin-angiotensin system activation or effects cannot be completely ruled out. Recently, it has been reported that the administration of triple therapy significantly reduced tubular and interstitial PCNA staining in unilateral ureteral obstruction, a hypertensive model characterized by locally activated renin-angiotensin system. Hydralazine, as part of the triple-therapy, may have some antiproliferative properties in situations characterized by high Ang II both directly [26] or through the inhibition of membrane-bound nicotinamide adenine dinucleotide phosphate (NADH) oxidase, a key enzyme in the Ang II-induced cell growth [35].

The molecular mechanisms of the increased apoptosis rate observed in spontaneously hypertensive rats with subtotal nephrectomy are unknown. We have observed an increment in the expression of the lethal protein Bax in the renal cortex of all animals with subtotal nephrectomy, which, as apoptosis, was mainly localized in tubular epithelial cells. Bax was noted to be increased in WKY rats with subtotal nephrectomy [36], as well as in other models of renal damage (reviewed in [5]). An association between increased apoptosis and overexpression of Bax protein has also been reported in the left ventricle of adult spontaneously hypertensive rats, which was normalized with the administration of the AT1 receptor antagonist losartan [31]. However, triple therapy was not studied by these authors.

The balance between the pro- and antiapoptotic Bcl-2 protein family members has been found to modulate apoptosis in many systems. In our study, Bcl-xL protein was increased in the renal cortex and tubular cells only in normotensive WKY rats + subtotal nephrectomy, raising a potential mechanism by which these animals could be protected against apoptosis. Up-regulation of antiapoptotic genes, such as Bcl-xL, in response to an adverse environment, has been noted in other models of tubular injury [37]. Interestingly, during nervous system ischemia failure to up regulate Bcl-x was a feature of damaged neurons, while it was increased in surviving neurons [38]. More recently, we have demonstrated that Bcl-xL overexpression protects from apoptosis induced by statins in murine tubular cells [39]. Strikingly, spontaneously hypertensive rats + subtotal nephrectomy failed to up-regulate tubular

Bcl-xL in response to renal mass reduction, leading to an increased Bax/Bcl-xL ratio. Failure to up-regulate Bcl-xL was associated with an enhanced apoptotic rate, when compared to WKY rats + subtotal nephrectomy. In a previous work, we have demonstrated that both Bcl-2 and Bcl-xL proteins are significantly lower both in glomeruli and mesangial cells in 8-week-old spontaneously hypertensive rats than in aged-matched WKY rats [40]. In contrast, Bax proteins were significantly higher in spontaneously hypertensive rats than in WKY rats. In our study, the administration of three antihypertensive therapies during 5 weeks to spontaneously hypertensive rats at the age of 9 weeks did not modify the Bcl-xL protein expression. Similarly, Liu et al [41] have reported a cardiac decreased Bcl-2 expression, but a increased Bax expression in spontaneously hypertensive rats. However, the administration of the ACE inhibitor ramipril to spontaneously hypertensive rats from 3 to 10 weeks of age resulted in a significant increase in Bcl-2 and a reduction in Bax. By contrast, Bcl-2 has been found to have a similar expression in left ventricle from WKY normotensive rats and spontaneously hypertensive rats at 30 weeks of age [32]. In addition, the chronic administration of losartan decreased Bax expression but did not modify the amount of Bcl-2 in the ventricles of spontaneously hypertensive rats. Thus, taken together, these data strongly suggest that apoptosis is regulated in a time- and organ-specific manner by antihypertensive drugs in vivo.

One major finding of our study was that blood pressure control did not result in increased anti-apoptosis Bcl-xL protein expression. This suggests that suppressed expression in Bcl-xL in spontaneously hypertensive rats could be independent of hypertension and subtotal nephrectomy and related to the genetic background. The cell signals that regulate Bcl-xL expression are unclear, although several transcription factors, including nuclear factor-κB (NF-κB), have recently been reported to directly regulate the Bcl-x gene [42]. Inhibition of NF-κB reduces Bcl-xL expression and sensitizes cells to TRAIL or [tumor necrosis factor-α (TNFα)]-induced apoptosis in different cell types [43, 44]. In vitro studies from our laboratory have demonstrated that mesangial cells obtained from hypertensive rats are more susceptible to TNF-α–induced apoptosis and they have less NF-κB activity than those obtained from normotensive rats [40]. Research currently under way in our laboratory is aimed at amplifying these findings and trying to solve the mechanisms responsible.

Our results, together with others in the literature, suggest that accelerated or excess apoptosis could contribute to the severity of renal lesions displayed by hypertensive, but not by normotensive rats, after subtotal nephrectomy. In this sense, at 5 weeks, spontaneously hypertensive rats with subtotal nephrectomy showed similar renal lesions to those found in normotensive rats with subtotal nephrectomy at day 90 in a previous paper [20]. In summary, at the time studied, subtotal nephrectomy induces in spontaneously hypertensive rats a greater activation of apoptotic mechanisms that could contribute to the earlier severity of renal damage observed in this model. Tight control of systolic blood pressure by either the administration of an AT1 receptor antagonist, an ACE inhibitor, or triple therapy was associated to the improvement of histologic lesions and the decrease in the apoptotic rate. Finally, our data suggest differences in the molecular mechanisms that control apoptotic cell death in hypertensive rats in comparison with normotensive rats.

ACKNOWLEDGMENTS

This work has been supported partially by grants from European Grant of Shared-cost RTD actions (QLG1-2002-1215), Comunidad de Madrid (08.4/0021/2003, 08.2/0030/2000), Fondo de Investigación Sanitaria (FIS) (01/3151 and 01/0199), Ministerio de Ciencia y Tecnología (SAF 2001/0717), Instituto de Investigaciones Nefrológicas "Reina Sofía," and Fundación Iñigo Álvarez de Toledo. R.L., J.G.-D., and M.P.C. are fellows from CAM, FIS and MEC respectively.

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