Long-term protective effect of UR-12670 after warm renal ischemia in uninephrectomized rats

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Background. The phospholipid platelet-activating factor (PAF) participates in the pathogenesis of renal ischemia/reperfusion injury, and *in vitro*, it induces synthesis of extracellular matrix proteins by mesangial and tubular epithelial cells. This study investigated the long-term effects of the potent orally active PAF antagonist UR-12670 in warm ischemic uninephrectomized rats, which was given according to different therapeutic schedules.

Methods. Uninephrectomized male Sprague-Dawley rats were divided into five groups and were followed for 52 weeks: rats without ischemia (SK); ischemic kidney for 60 minutes (SIK); ischemic kidney and UR-12670 from 0 to the 7th day (UR 0-7); ischemic kidney and UR-12670 from day 0 to 52 weeks (UR 0-E); and ischemic kidney and UR-12670 from day 8 to week 52 (UR 8-E). Two more groups (ischemic and UR treated) served to evaluate the UR-12670–protective effect on ischemic acute renal failure at one week.

Results. UR-12670 administration exerted functional and morphological protection against post-ischemic acute renal failure. The ischemic untreated (SIK) group developed progressive proteinuria from week 12. The onset of proteinuria in ischemic UR-12670–treated groups was delayed to the 24th week, and it was significantly lower than in SIK group throughout the study. Only SIK and ischemic-treated UR 0-7 rats presented with chronic renal failure, as shown by creatinine, creatinine clearance, glomerular filtration rate (GFR), and renal plasma flow (GFR 52 weeks: SK, 2525 ± 267; SIK, 992 ± 149; UR 0-7, 1551 ± 385 μl/min). Kidneys from the short-term treated group (UR 0-7) showed a reduction of glomerulosclerosis (SK, 14.4 ± 3.7; SIK, 75.7 ± 7.7; UR 0-7, 41.5 ± 8.5%) and vascular myointimal hyperplasia, but the tubulointerstitial damage (tubulointerstitial score: SK, 0.2 ± 0.2; SIK, 4.4 ± 0.5; UR 0-7, 3.7 ± 0.7) was similar to that in the ischemic untreated group. Long-term ischemic treated rats (UR 0-E, UR 8-E) did not develop chronic renal failure (GFR: UR 0-E, 2059 ± 314; UR 8-E, 2410 ± 208 μl/min). In these groups, glomerulosclerosis (UR 0-E, 32.8 ± 5.8; UR 8-E, 24.3 ± 3.0%), tubulointerstitial damage (tubulointerstitial score: UR 0-E, 2.1 ± 0.5; UR 8-E, 1.9 ± 0.3) and vascular myointimal hyperplasia were significantly lower than in the ischemic untreated group. By *in situ* hybridization, an increase of transforming growth factor-β1 mRNA expression in glomerular and tubular cells was observed in ischemic untreated and ischemic treated UR 0-7 rats. UR-12670 long-term treated rats showed a clear reduction of transforming growth factor-β1 mRNA-positive glomerular cells.

Conclusion. The chronic administration of the PAF antagonist UR-12670 attenuates the long-term effects of ischemia-reperfusion injury in uninephrectomized rats. The beneficial effect of this agent, even when given beyond the initial ischemia/reperfusion injury, suggests that PAF plays a role in the mechanisms of progression to late renal damage in this model.

Chronic transplant nephropathy, defined by progressive functional deterioration associated with interstitial fibrosis, tubular atrophy, and glomerulosclerosis, is the leading cause of late renal allograft failure [1]. Risk factors for chronic transplant nephropathy have been considered as antigen-dependent (that is, immunological) and antigen-independent (non-immunological) factors [2]. The antigen-independent factor ischemia/reperfusion injury and its clinical expression, delayed graft function, seem to exert a negative effect on graft survival [3, 4]. In a model of warm renal ischemia combined with contralateral nephrectomy, it has been demonstrated that ischemia/reperfusion injury induces long-term chronic lesions that mimic those encountered in chronic transplant nephropathy [5]. It has been proposed that when only one kidney is engrafted, ischemia/reperfusion injury causes a further reduction in total nephron mass that is sufficient to accentuate glomerular injury. Surviving nephrons undergo hyperfiltration and hypertrophy, which are associated with glomerular capillary hypertension,

synthesis of cytokines and growth factors, and extracellular matrix accumulation, which lead to glomerulosclerosis [6]. Thus, it is well known that in mesangial cells, mechanical stress leads to matrix accumulation by means of the induction of transforming growth factor-β1 (TGF-β1) [7], which is recognized as a key fibrogenic cytokine involved in chronic transplant nephropathy [8].

Platelet-activating factor (PAF) is a potent phospholipid mediator involved in acute inflammatory and immune responses [9]. Our group has previously shown that treatment with PAF receptor antagonists protects from warm renal [10] and cold [11] ischemia/reperfusion injury in rat kidney, as well as from delayed graft function in cadaveric kidney transplantation [12]. However, the role of PAF in the development of chronic nephropathy induced by ischemia/reperfusion injury has not been previously studied. Interestingly, it has been recently described that PAF induces the synthesis of extracellular matrix proteins by mesangial [13] and tubular epithelial cells [14], suggesting a role for PAF in glomerulosclerosis and interstitial fibrosis.

The aim of this work was to evaluate, in the absence of allorecognition, whether or not the administration of the oral PAF receptor antagonist UR-12670 could prevent the development of chronic nephropathy induced by warm renal ischemia/reperfusion injury associated with nephron mass reduction. We also evaluated whether this effect could be due to the protection against post-ischemic acute renal failure, by giving a short course of UR-12670, or to the interference in PAF-mediated mechanisms potentially involved in long-term tissue remodeling and fibrosis, by a chronic treatment with UR-12670.

METHODS

Experimental design

Male Sprague-Dawley rats (250 to 300 g body wt) were used in this study. Animal care and interventions were conducted in accordance with the guidelines of the European Community Committee on care and use of laboratory animals and good laboratory practice. After intramuscular anesthesia with a mixture of ketamine (75 mg/kg), atropine (0.05 mg/kg), and diazepam (5 mg/kg), the animal was placed on a warming pad in order to maintain body temperature at the physiological range. A medial laparotomy was performed, and left kidney normothermic ischemia was induced by renal pedicle occlusion for 60 minutes using a nontraumatic clamp. Simultaneously, right nephrectomy was done, and finally, reperfusion was confirmed visually after clamp removal. Then the animals were housed in a room kept at constant temperature with a 12-hour dark/12-hour light cycle. Rats had free access to tap water and were fed a standard rodent diet.

Acute experiments were performed to assess the protective effect of oral UR-12670 against post-ischemic acute renal failure. Two groups of animals were studied according to the experimental procedure mentioned earlier in this article. Rats were followed for seven days and on the first, second, third, and seventh day, 0.5 ml of blood was obtained from the tail vein for serum creatinine measurement. Conventional histology was performed on day 7. One of the groups was a control (ischemic, $N = 11$), and the other received oral UR-12670 daily (UR-treated, $N = 10$).

For chronic experiments, animals were divided into five groups. Two were control groups: (*a*) the single kidney group (SK), uninephrectomized rats with a left non-ischemic kidney ($N = 7$), and (*b*) the single ischemic kidney group (SIK), uninephrectomized rats with left warm renal ischemia ($N = 12$). (*c*) One was a short-term treatment group of uninephrectomized rats with left warm renal ischemia and UR-12670 administration from day 0 until the seventh day post-ischemia and then followed for 52 weeks (UR 0-7; $N = 12$). Finally, two were long-term UR-12670–treated groups: (*d*) uninephrectomized rats with left warm renal ischemia and UR-12670 administration from day 0 until the 52nd week (UR 0-E; $N = 10$), and uninephrectomized rats with left warm renal ischemia in which drug administration was delayed for eight days after ischemia and then given until the 52nd week (UR 8-E; $N = 12$). Additional rats were evaluated in the SK, SIK, and UR 0-E groups to allow histological studies at 16 and 32 weeks ($N = 5$ rats each).

Before the experiment and every four weeks after the surgical procedure, all animals were placed in metabolic cages in order to collect a 24-hour urine sample. Animals were weighed at the same intervals, and 0.5 ml of blood from the tail vein was obtained.

Platelet-activating factor antagonist administration

UR-12670 (Uriach Laboratories, Barcelona, Spain) is a potent, orally active, and highly selective PAF receptor antagonist that has been tested in several *in vivo* models [15, 16]. The chemical name of UR-12670 is 1-[[1-(3,3-diphenylpropanoyl)-4-piperidyl]methyl]-1-H-2-methyl-imidazo [4,5-c] pyridine. It has a molecular weight of 438.57 and is a hydrosoluble compound with a 100% oral bioavailability [15].

UR-12670 was administered intravenously at 10 mg/kg five minutes before reperfusion and was later given orally at 20 mg/kg by daily gavage.

Biochemical data

Proteinuria (mg/24 hr) was determined every four weeks by the Ponceau method (Bayer Diagnósticos, Madrid, Spain). Serum and urine creatinine (mg/dl) were determined in the same intervals by Jaffe's reaction on an autoanalyzer (Beckman Instruments, Palo Alto, CA, USA), and creatinine clearance (ml/min/100 g body wt) was calculated by the standard formula. At 12, 36, and

52 weeks, total serum cholesterol was measured by an enzymatic colorimetry method on an autoanalyzer (Beckman Instruments).

Renal function studies

At 52 weeks, animals were subjected to invasive functional studies to assess glomerular filtration rate and renal plasma flow and to measure arterial pressure. For this purpose, rats were anesthetized again with an intramuscular mixture of ketamine/diazepam/atropine and were placed on a heating pad to maintain body temperature at a physiological range. Vascular polyethylene catheters (PE-50) were implanted into the right carotid artery and right jugular vein. Arterial catheter was used for continuous monitoring of arterial pressure by means of an electronic pressure transducer (Nihon Kohden Co., Germany) and for blood sampling. A venous catheter was used for the infusion of fluid and clearance markers. The abdominal cavity was opened, and the ureter was cannulated for the collection of urine with polyethylene tubing (PE-10). Rats received a priming load of inulin (Polyfructosan; Laevosan, Linz, Austria) and paraaminohippuric acid (PAH; Merck, Darmstadt, Germany) (83 mg of inulin and 5 mg of PAH) via the right jugular vein, followed by a continuous infusion of a solution containing 2% inulin and 0.6% PAH at a constant rate of 0.5 ml/hr/100 g body wt. Urine collection was started after an equilibration period of 60 minutes. Three periods of 20 minutes were established to determine inulin and PAH clearances. At the midpoint of each period, 0.5 ml of arterial blood was obtained. The inulin concentration in plasma and urine was determined by the indole-3-acetic acid (Merck) colorimetric assay. The PAH concentration in plasma and urine was measured by the colorimetric Branson method. Glomerular filtration rate (GFR) and renal plasma flow (RPF), measured as inulin and PAH clearances, respectively, were calculated by standard formulas, and the final result was the mean of the three values from each 20-minute period. It is well known that in chronic renal disease the PAH extraction ratio is reduced, and thus, PAH clearance may underestimate true RPF in this setting. The absolute values of PAH clearance should therefore be considered with the caveat that a systematic error may exist. Nevertheless, PAH clearance was used as a comparative index of the putative changes in RPF occurring with each experiment. At the end of the study, kidneys were perfused with a 4°C isotonic saline solution, removed, weighed, and processed for tissue studies.

Light microscopy

Coronal 1 to 2 mm thick slices of the kidney were fixed in 4% formaldehyde and embedded in paraffin, and 3 to 4 μm thick tissue sections were stained with hematoxylin and eosin, periodic acid-Schiff, silver me-thenamine, and Masson trichrome. Light microscopic sections were reviewed by a pathologist blinded to the treatment groups.

In acute experiments, sections were examined for several parameters of tubulointerstitial damage, including tubular dilation, tubular cell death and detachment, dystrophic calcifications, and interstitial edema and acute inflammation. For each case, a global estimation of the tubular lesion was made and semiquantitatively graded on a scale in which 0 was no abnormality, and 1+, 2+, 3+, and 4+ represented slight, mild, moderate, and severe abnormalities, respectively.

In chronic experiments, glomerulosclerosis was assessed by examining at least 100 glomeruli in each kidney and was expressed as the percentage of glomeruli presenting either segmental or global sclerosis and hyalinosis. Tubular (atrophy and dilation) and interstitial changes (fibrosis and mononuclear cell infiltrate), as well as arterial myointimal hyperplasia, were graded from 0 to 3+ (0, no changes; 1+, changes affecting less than one third of the sample; 2+, changes affecting between one and two thirds of the sample; 3+, changes affecting more than two thirds of the sample). In order to summarize the tubulointerstitial changes in each animal, a tubulointerstitial score was calculated by adding up the individual value of the tubular damage, interstitial fibrosis, and interstitial cell infiltrate.

In situ transforming growth factor-β1 hybridization

Riboprobe for rat TGF-β1 (298 pb; Clontech Laboratories, Palo Alto, CA, USA) was prepared by the reverse transcription polymerase chain reaction (RT-PCR) using the 5′-primer, 5′-C TTC AGC TCC ACA GAG AAG AAC TGC-3′, and 3′-primer, 5′-CAC GAT CAT GTT GGA CAA CTG CTC C-3′.

Reverse transcription-PCR was performed with RNA extracted from rat kidney serving as the template in a Thermal Cycler (Perkin Elmer Corp., Norwalk, CT, USA). Total RNA was obtained by the acid guanidine-thiocyanate-phenol-chloroform method [17], and 1 μg of RNA was reverse transcribed to single-stranded cDNA by incubation with reverse transcription mixture [5 mM MgCl$_2$, 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 1 mM dNTPs mixture, 50 ng oligo(dT) primer, 20 U RNasin, and 15 U reverse transcriptase from avian myeloblastosis virus; all from Promega, Madison, WI, USA]. Each PCR reaction containing 20 pmol of primers, 0.5 μCi [α^{32}P]dCTP (3000 Ci/mmol; Amersham, Arlington Heights, IL, USA) and 3 U Taq DNA polymerase was carried out under the following conditions: denaturing at 94°C for one minute, annealing at 60°C for one minute, and elongating at 72°C for two minutes. The expression of glyceraldehyde-3-phosphate dehydrogenase was used as internal control. The PCR product was ligated into a PCR3 vector (Invitrogen, San Diego, CA, USA).

Orientation was confirmed by sequence and restriction digestion. The templates were linearized with appropriate restriction enzymes, and then labeled antisense and sense cRNA probes were generated using T7 or SP6 polymerases and digoxigenin-labeled uridine-triphosphate (Boehringer Mannheim, Mannheim, Germany) as substrate according to the manufacturer's instructions.

For *in situ* hybridization, five randomly selected paraffin-embedded tissue sections per group were fixed in 1.5% paraformaldehyde-1.5% glutaraldehyde for 10 minutes, washed and subsequently treated with 5 mM levamisole, 0.2 N HCl, and 25 µg/ml proteinase K in 0.1 M Tris pH 7.8, 5 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% sodium dodecyl sulfate (SDS). Hybridization was carried out at 42°C with 0.4 ng/µl denatured digoxigenin-labeled riboprobes in hybridization solution [2 × standard saline citrate (SSC), 1 × Denhardt's, 0.1 M sodium phosphate pH 6.5, 10% dextran sulfate, 40% deionized formamide, 24 mM Vanadyl Ribonuclease complex, and 0.5 mg/ml yeast tRNA]. After washing in 2 × SSC for five minutes and 0.2 × SSC for three minutes, sections were incubated with alkaline phosphatase conjugated antidigoxigenin antibody (1:750; Boehringer Mannheim). Colorimetric detection of mRNA was performed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate in dark for 10 to 30 minutes. Negative controls consisted of matched serial sections hybridized without RNA probe, with sense probe and sections pretreated with 25 µg/ml RNAse A for 20 to 30 minutes before hybridization with the corresponding antisense probe.

We separately evaluated the mRNA TGF-β1 expression in glomeruli and tubules. The total number of positive glomerular cells was counted in 30 glomeruli, and the mean value was used as a glomerular index of TGF-β1 (number of positive cells/glomerulus). The extent of tubular staining was assessed by means of a semiquantitative scale grading from 0 to 3+.

Statistical analysis

To compare the two groups, in acute experiments, statistical analysis was performed by the U-Mann Whitney method. In long-term experiments, the chi-square test was used to compare the mortality ratio from acute renal failure between the treated and nontreated groups. To compare more than two groups for proteinuria, serum creatinine, and creatinine clearance throughout the entire follow-up period, statistical analysis was performed by the one-way analysis of variance (ANOVA) for repeated-measures method. To determine the origin of the differences at any time point for these parameters, we used the one-way ANOVA for independent values followed by Scheffe's test. To compare histological and *in situ* TGF-β1 mRNA data, we used the nonparametric Kruskal–Wallis test for between-subject analysis fol-

Table 1. Renal functional profile (Creatinine, µmol/liter) and histologic evaluation of the acute tubulointerstitial lesions in Ischemic group ($N = 8$; sixty minutes of left kidney warm ischemia, contralateral nephrectomy and seven days of follow-up), and in the UR-treated group ($N = 8$; the same experimental method plus the administration of daily oral UR-12670)

	Ischemic	UR-treated	*P*
Serum creatinine			
Basal	54 ± 4	55 ± 3	0.75
First day	295 ± 16	308 ± 19	0.85
Second day	392 ± 34	288 ± 30	0.04
Third day	366 ± 44	195 ± 22	0.004
Seventh day	79 ± 10	59 ± 2	0.08
Histological damage	3.2 ± 0.9	1.9 ± 0.8	0.006

lowed by *a posteriori* individual comparisons by Connover's test. To analyze the correlation between proteinuria and cholesterol, the simple regression analysis was used.

All *P* values were two tailed, and a *P* value of <0.05 was considered statistically significant. Data are presented as mean ± SEM.

RESULTS

Effect of UR-12670 on ischemic acute renal failure

This effect was analyzed in two groups of warm ischemic uninephrectomized rats (ischemic vs. UR-treated groups) followed for seven days. The mortality rate in this set of acute experiments was 3 out of 11 in the ischemic group and 2 out of 10 in the UR-treated group. Thus, eight animals per group fulfilled the seven-day follow-up. Twenty-four hours after ischemia, both groups of rats displayed a similar degree of renal failure, as shown by the serum creatinine level (Table 1). Nontreated rats showed a further increase in serum creatinine that peaked on second day. Conversely, UR-12670–treated animals showed a progressive decline in serum creatinine on the second and third days. In fact, significant differences between groups were observed on the second and third days. Seven days after ischemia, the serum creatinine level returned to the basal value only in UR-treated rats, although no significant differences between both groups were observed.

Concerning the histological evaluation, UR-treated animals showed a significantly lower acute tubulointerstitial lesions induced by ischemia/reperfusion injury than ischemic animals (Table 1).

Long-term effect of UR-12670 on renal functional parameters after warm renal ischemia in uninephrectomized rats

Considering all of the animals included for chronic follow-up studies, the mortality ratio from acute renal failure in the first week after ischemia was higher in rats that did not receive UR-12670 during this period
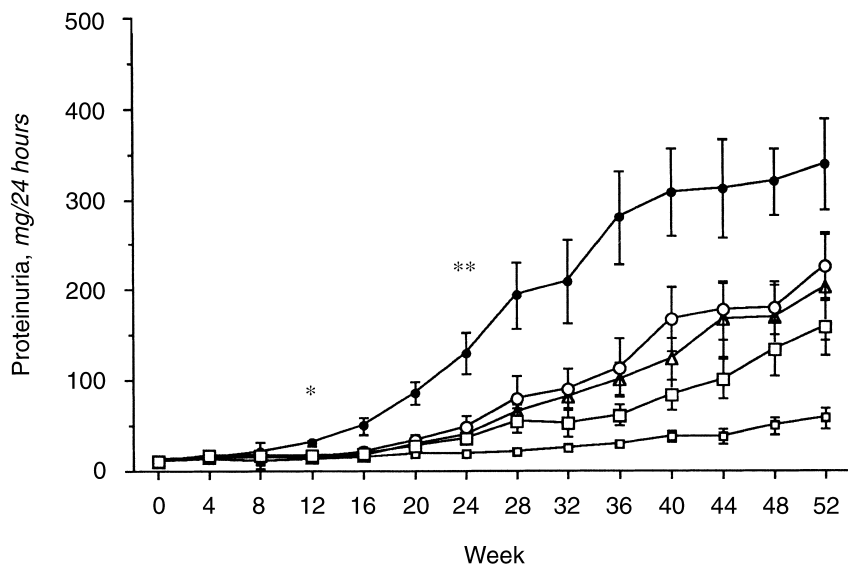
Fig. 1. Effect of UR-12670 on proteinuria after warm renal ischemia in uninephrectomized rats. Symbols are: (□; SK) single kidney, $N = 7$; (●; SIK) single ischemic kidney, $N = 9$; (△; UR 0-7) UR-12670 treated from 0 to 7 days, $N = 10$; (□; UR 8-E) UR-12670 treated from the 8th day until 52 weeks, $N = 8$; (○; UR 0-E) UR-12670 treated from 0 to 52 weeks, $N = 9$. Uninephrectomized ischemic rats (SIK) developed progressive proteinuria from the 12th week (*$P < 0.0001$ SIK vs. the other groups). The onset of proteinuria in UR-12670–treated groups (UR 0-7, UR 0-E, and UR 8-E) was delayed to the 24th week (**$P = 0.04$, UR-12670 treated groups vs. SIK). By the one-way ANOVA for the repeated-measures method, proteinuria was higher in the SIK group than in the SK ($P < 0.0001$), UR 0-7 ($P < 0.0001$), UR 0-E ($P < 0.0001$), and UR 8-E ($P < 0.0001$) groups. Also, proteinuria was lower in the SK group than in UR 0-7 ($P = 0.0006$), UR 0-E ($P < 0.0001$), and UR 8-E ($P = 0.02$) groups. No significant differences were seen among the three UR-12670–treated groups.

(SIK$_{16+32+52}$ and UR 8-E$_{52}$, $N = 39$) than in those animals to which the PAF antagonist was administered (UR 0-7$_{52}$ and UR 0-E$_{16+32+52}$, $N = 36$, 35.9 vs. 13.9%, respectively, $P = 0.03$). No further mortality was observed during the follow-up periods in any of the groups.

After recovering from acute renal failure, the number of animals followed for 52 weeks was as follows: SK, $N = 7$; SIK, $N = 9$; UR 0-7, $N = 10$; UR 8-E, $N = 8$; UR 0-E, $N = 9$. In the SIK group, proteinuria appeared in the 12th week and showed a progressive increase throughout the entire follow-up (Fig. 1). In UR-12670–treated groups, proteinuria onset was delayed until the 24th week, and it was significantly lower than in the SIK group throughout the experiment. There were no differences among the three treated groups at any time point or along the entire follow-up. The SK group showed only mild proteinuria, which was similar to that in UR-12670 treated groups until the 40th week.

The serum creatinine level in the SIK group showed a slight and progressive increase from the 28th week, which became significantly higher beyond the 40th week when compared with SK and UR 8-E groups (Fig. 2). From week 48, significant differences were found between SIK and SK and both long-term treated groups (UR 0-E and UR 8-E). In week 52, the SIK group showed severe renal failure with a mean serum creatinine of 2.2 ± 0.7 mg/dl. As shown in Figure 2, only SIK and UR 0-7 rats developed chronic renal failure. Conversely, both long-term treated groups (UR 0-E and UR 8-E) showed similar serum creatinine values to those of SK group. Differences among groups in creatinine clearance throughout the study, as well as GFR and RPF values obtained at the end of the experiment, paralleled those of serum creatinine (Table 2). No differences were found in inva-

sive arterial pressure among all of the experimental groups, although potentially, anesthesia may have modified this measure.

The total serum cholesterol showed a progressive increase in the SIK group throughout the experiment, although it remained unchanged in the rest of the groups (Table 2). A strongly positive correlation was seen between proteinuria in the 12th week and total serum cholesterol in the 36th and 52nd weeks ($P < 0.0001$, $r^2 = 0.5$, and $P < 0.0001$, $r^2 = 0.49$, respectively).

Effect of UR-12670 on long-term renal morphology after warm renal ischemia in uninephrectomized rats

When analyzing histological parameters, the SIK group showed a significantly higher percentage of global glomerulosclerosis compared with SK (Table 3). All UR-12670–treated groups showed a lower percentage of global glomerulosclerosis than SIK. In the UR 0-E group, these differences began in the 16th week, were maintained in the 32nd week, and became statistically significant in the 52nd week. When we extended the glomerulosclerosis quantitation to not only global glomerular alterations but also focal and segmental glomerulosclerosis, UR-12670–treated groups also showed significantly lower percentages than SIK. Nevertheless, when compared with SK, the UR 0-7 group displayed significantly higher global and segmental glomerulosclerosis. On the contrary, in both long-term treated groups, glomerulosclerosis was not different from that in SK.

When tubular atrophy, interstitial cell infiltrate, and fibrosis were analyzed, similar differences were observed between groups. In 16th and 32nd week, kidneys from the SIK group showed some degree of tubulointerstitial injury, which was obvious in 52nd week (Fig. 3A). As
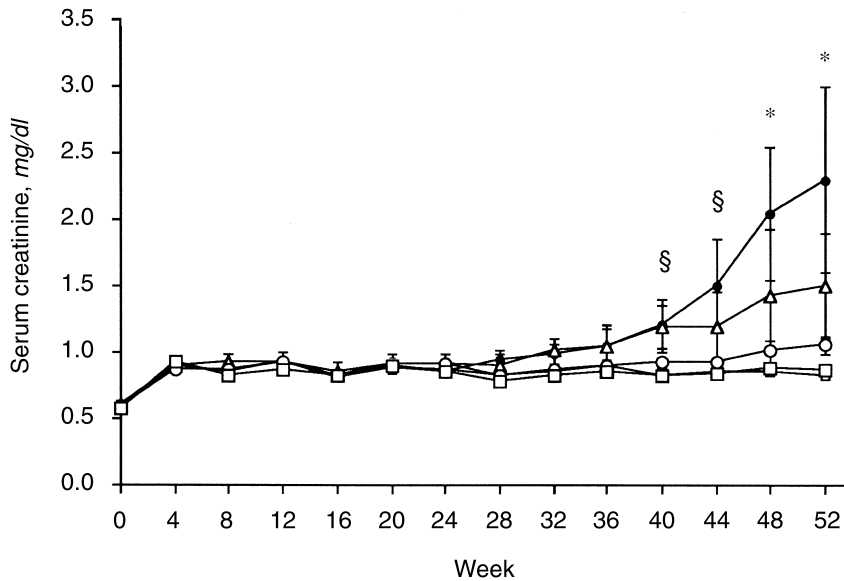
Fig. 2. Effect of UR-12670 on serum creatinine after warm renal ischemia in uninephrectomized rats. Symbols are: (■; SK) single kidney, $N = 7$; (●; SIK) single ischemic kidney, $N = 9$; (△; UR 0-7) UR-12670 treated from 0 to 7 days, $N = 10$; (□; UR 8-E) UR-12670 treated from the 8th day until 52 weeks, $N = 8$; (○; UR 0-E) UR-12670 treated from 0 to 52 weeks, $N = 9$. In the SIK group, serum creatinine increased progressively from the 28th week, and it became significantly higher (§) beyond the 40th week when compared with SK ($P = 0.04$) and UR 8-E ($P = 0.04$) groups. From the 48th week, significant differences (*) were found between the SIK and SK groups ($P = 0.03$) and both long-term treated groups (UR 0-E, $P = 0.04$; UR 8-E, $P = 0.02$). By the one-way ANOVA for repeated-measures method, serum creatinine was higher in the SIK group than in the SK ($P = 0.0001$), UR 0-E ($P = 0.0009$) and UR 8-E ($P < 0.0001$) groups, and was similar to that in UR 0-7 group ($P = 0.3$). Also, serum creatinine was higher in UR 0-7 group than in the UR 0-E ($P = 0.04$), UR 8-E ($P = 0.008$), and SK ($P = 0.01$) groups. No differences were observed between the SK group and the UR 0-E ($P = 0.5$) and UR 8-E ($P = 0.9$) groups.

Table 2. Total serum cholesterol, renal functional parameters, arterial pressure and kidney weight in all of the study groups

	Week	SK $N = 7$	SIK $N = 9$	UR 0-7 $N = 10$	UR 0-E $N = 9$	UR 8-E $N = 8$
Total serum cholesterol, *mg/dl*	12	70 ± 4	68 ± 4	61 ± 2	61 ± 2	66 ± 6
	36	73 ± 5	$179 \pm 6^{a,b}$	96 ± 8	88 ± 5	77 ± 9
	52	86 ± 10	$187 \pm 4^{a,b}$	109 ± 1	108 ± 1	105 ± 1
Creatinine clearance *μl/min/100 g*	16	287 ± 14	271 ± 14	272 ± 18	278 ± 10	300 ± 12
	32	301 ± 15	278 ± 15	283 ± 39	308 ± 15	314 ± 10
	52	259 ± 14	$166 \pm 30^{a,c}$	203 ± 32^{d}	228 ± 15	270 ± 13
Glomerular filtration rate *μl/min*	52	2525 ± 267	$992 \pm 149^{a,c}$	1551 ± 385^{d}	2059 ± 314	2410 ± 208
Renal plasma flow *μl/min*	52	8223 ± 1064	$3815 \pm 680^{a,c}$	5340 ± 1285^{d}	6951 ± 678	7419 ± 394
Arterial pressure *mm Hg*	52	116 ± 3	121 ± 5	126 ± 3	118 ± 4	127 ± 4
Kidney weight *g*	52	2.8 ± 0.1	$4.1 \pm 0.2^{a,c}$	3.4 ± 0.4	3.2 ± 0.1	2.9 ± 0.2

Statistical analysis was performed by one-way analysis of variance followed by Scheffé's test. Abbreviations are: SK, single kidney; SIK, single ischemic kidney; UR-07, short-term UR-12670 treated group from day 0 to day 7; UR 0-E, long-term UR-12670 treated group from day 8 to 52 weeks.

[a] $P < 0.05$ SIK vs. SK
[b] $P < 0.05$ SIK vs. UR 0–7, UR 0-E, UR 8-E
[c] $P < 0.05$ SIK vs. UR 0-E, UR 8-E
[d] $P < 0.05$ UR 0–7 vs. SK

shown in Table 3, kidneys from SIK group exhibited a higher level of tubulointerstitial damage than kidneys from the SK group, in which only minimal changes were seen. When kidneys from the SIK group were compared with the UR 0-7 group (Fig. 3B), no significant differences were found (tubulointerstitial score: SIK, 4.4 ± 0.5; UR 0-7, 3.7 ± 0.7, $P = 0.35$). Conversely, both long-term treatment groups (Fig. 3C) showed significantly lower tubular atrophy, interstitial infiltrate, and fibrosis than the SIK group (Table 3).

Kidneys from the SK group showed no vascular abnormalities throughout the follow-up. On the contrary, in the 32nd week and especially in the 52nd week, kidneys from the SIK group had vascular myointimal hyperplasia with luminal narrowing of the affected vessels. Interest-

ingly, kidneys from UR-12670–treated groups, in short and long-term schedules, had significantly fewer vascular lesions than kidneys from the SIK group.

Effect of UR-12670 on renal TGF-β1 mRNA expression

The SIK group showed higher TGF-β1 mRNA expression in glomerular (Figs. 3D and 4A) and tubular (Figs. 3D and 4B) cells than the SK group. It is noteworthy that tubular staining disclosed focal distribution (Fig. 3D). When analyzing UR-12670–treated groups, the TGF-β1 mRNA glomerular index was similar in UR 0-7 and SIK (Figs. 3E and 4A) animals. In both long-term UR-12670–treated groups (Fig. 3F), the number of positive glomerular cells was significantly lower than in the

Table 3. Glomerulosclerosis, vascular myointimal hyperplasia and chronic tubulointerstitial parameters at 16, 32 and 52 weeks

	Week	SK	SIK	UR 0–7	UR 0-E	UR 8-E
Global glomerulosclerosis %	16	1.0 ± 0.6	4.9 ± 1.5[a]		2.7 ± 0.9	
	32	2.4 ± 1.0	12.6 ± 3.1[a]		5.7 ± 3.2	
	52	1.7 ± 0.5	36.1 ± 8.3[a,b]	12.4 ± 3.1	14.7 ± 4.5	9.9 ± 2.9
Global + segmental glomerulosclerosis %	52	14.4 ± 3.7	75.7 ± 7.7[a,b]	41.5 ± 8.5[c]	32.8 ± 5.8	24.3 ± 3.0
Tubular atrophy	16	0 ± 0	0.5 ± 0.3		0.7 ± 0.2	
	32	0.2 ± 0.2	1.2 ± 0.4[a]		0.6 ± 0.2	
	52	0.2 ± 0.2	1.8 ± 0.2[a,d]	1.5 ± 0.3	0.9 ± 0.2	0.9 ± 0.1
Fibrosis	16	0 ± 0	0.3 ± 0.3		0 ± 0	
	32	0 ± 0	0.4 ± 0.2		0 ± 0	
	52	0 ± 0	1.1 ± 0.2[a,d]	0.8 ± 0.3[e]	0.5 ± 0.2	0.3 ± 0.2
Interstitial infiltrate	16	0 ± 0	1.0 ± 0.4[a]		0.7 ± 0.2	
	32	0 ± 0	1.0 ± 0.3[a]		0.8 ± 0.2	
	52	0 ± 0	1.5 ± 0.2[a,d]	1.3 ± 0.2[e,f]	0.7 ± 0.2	0.7 ± 0.2
Tubulointerstitial score	16	0 ± 0	1.8 ± 0.8[a]		1.5 ± 0.5	
	32	0.2 ± 0.2	2.6 ± 0.8[a]		1.4 ± 0.4	
	52	0.2 ± 0.2	4.4 ± 0.5[a,d]	3.7 ± 0.7[e]	2.1 ± 0.5	1.9 ± 0.3
Vascular myointimal hyperplasia	16	0 ± 0	0 ± 0		0 ± 0	
	32	0 ± 0	1.0 ± 0.2[a]		0.4 ± 0.3	
	52	0 ± 0	1.2 ± 0.1[a,b]	0.7 ± 0.2	0.5 ± 0.2	0.2 ± 0.2

Abbreviations are in Table 2. The number of animals at 16 and 32 weeks was 5 in each experimental group (SK, SIK and UR 0-E). At 52 weeks: SK, $N = 7$; SIK, $N = 9$; UR 0–7, $N = 10$; UR 8-E, $N = 8$; UR 0-E, $N = 9$. Statistical analysis was performed by Kruskall-Wallis test and *a posteriori* individual comparisons by Connover's test.

[a] $P < 0.05$ SIK vs. SK
[b] $P < 0.05$ SIK vs. UR 0–7, UR 0-E, UR 8-E
[c] $P < 0.05$ UR 0–7 vs. SK
[d] $P < 0.05$ SIK vs. UR 0-E, UR 8-E
[e] $P < 0.05$ UR 0–7 vs. UR 8-E
[f] $P < 0.05$ UR 0–7 vs. UR 0-E

SIK group and similar to the SK group (Fig. 4A). In contrast, there were no significant differences in tubular staining between all treated groups and kidneys from the SIK group (Fig. 4E).

DISCUSSION

In this work, we have shown that the administration of the PAF receptor antagonist UR-12670 attenuates the long-term effects of ischemia/reperfusion injury in uninephrectomized rats. Animals chronically receiving UR-12670 have a delay in the onset and a reduction of proteinuria, do not develop chronic renal failure, and have a decrease in the glomerular TGF-β1 mRNA expression, as well as in the glomerulosclerosis and interstitial fibrosis. This study also shows that protection from post-ischemic acute renal failure by a PAF antagonist delays and reduces proteinuria, but does not completely hamper the appearance of chronic renal failure and interstitial fibrosis. The beneficial effect of UR-12670, even when given beyond the first week, that is, after the initial ischemic injury has already occurred, strongly suggests that PAF plays a role in the mechanisms of progression of chronic renal damage.

There is some evidence suggesting that the nonimmunological factor ischemia/reperfusion injury may be involved in the pathogenesis of chronic transplant nephropathy [4, 18]. In renal transplantation, a 50% nephron mass is usually offered because only one kidney is grafted. In this context, it has been proposed that further loss of nephrons because of initial ischemia may be critical [5, 19], and thus, these kidneys develop chronic renal dysfunction by mechanisms described in the so-called hyperfiltration theory [20, 21]. Thus, models of a single kidney subjected to either warm or cold ischemia in uninephrectomized rats are valuable tools to explore antigen-independent mechanisms of chronic transplant nephropathy [4, 22, 23].

The expression pattern of cellular and molecular mediators in the early [22–24] and late [5] stages after ischemia/reperfusion injury has been previously reported. Several groups, including ours [10, 11, 25], have recently described that PAF also plays a key role in the regulatory mechanisms of post-ischemic acute renal failure. In previous studies, we showed that a single intravenous injection of the PAF receptor antagonist, BN 52021, prevented renal dysfunction and histological damage caused by warm ischemia. Furthermore, in a model of cold ischemia, BN 52021 was able to overcome the functional damage induced by PAF [11]. Because UR-12670 has not been tested in warm renal ischemia, we started our study by analyzing the protection of renal ischemia/reperfusion injury by this agent. Results from the acute experiments using UR-12670 extend the observations mentioned in this section, showing that PAF receptor blockade attenuates the renal dysfunction and, even more interesting, the histological lesions induced by warm ischemia.
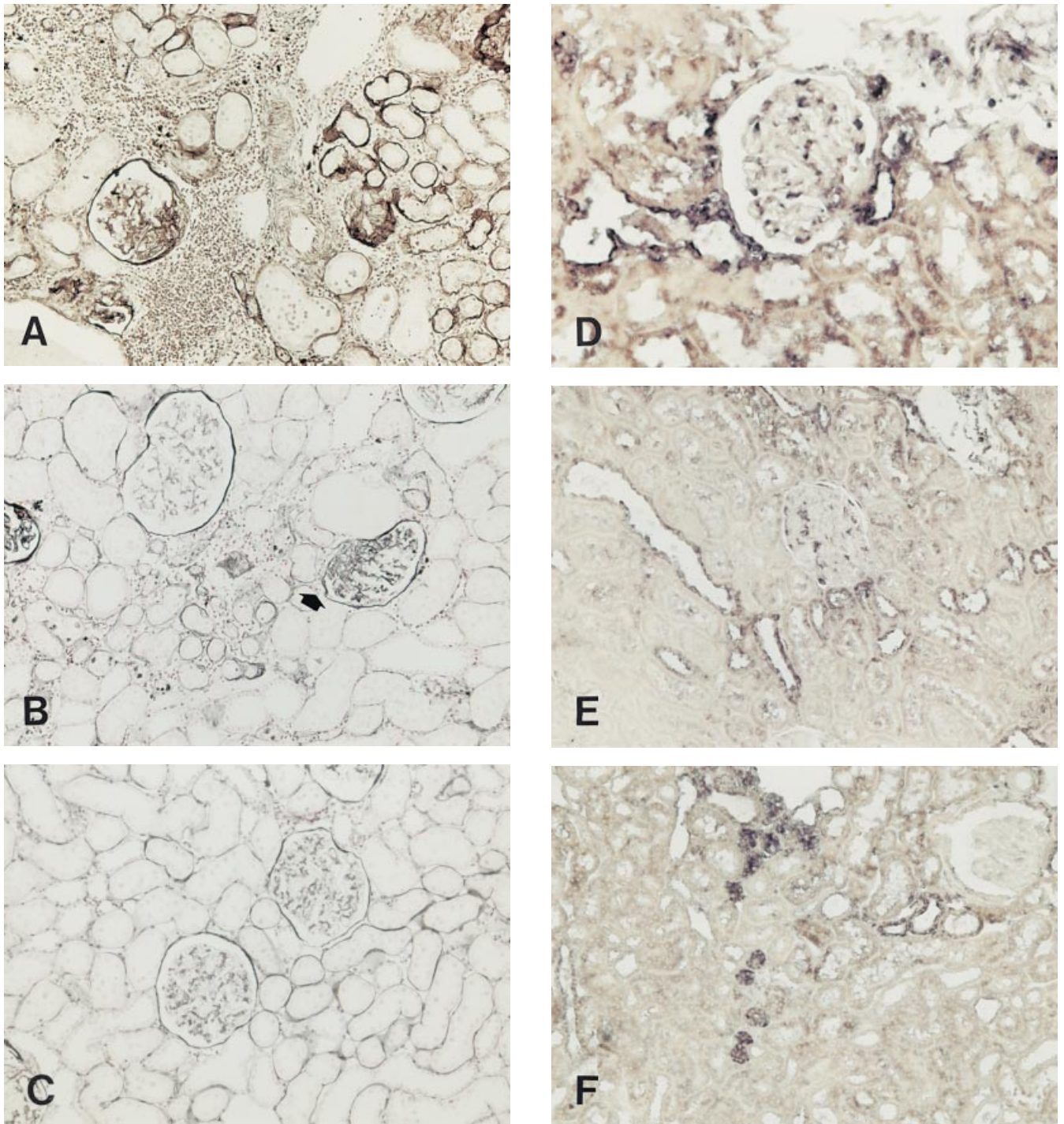
The long-term impact of these initial functional and

Fig. 3. Silver methenamine staining (*A–C*) and *in situ* hybridization of TGF-β1 mRNA (*D–F*) in kidneys from SIK, UR 0-7, and UR 0-E groups harvested at 52 weeks. (A) A representative kidney section from SIK group showing glomerulosclerosis, severe interstitial cell infiltrate, and tubular atrophy and dilation (×200). (B) Tubulointerstitial lesions are evident in kidneys from UR 0-7 group. One glomerulus shows focal and segmental glomerulosclerosis (arrow; ×200). (C) Mild tubulointerstitial lesions and glomerulosclerosis were observed in UR-12670 long-term treated groups, as shown by this representative kidney section from the UR 0-E group (×200). (D) Kidney from a SIK rat showing groups of tubules with strong cytoplasmic TGF-β1 mRNA expression. Several positive glomerular cells are also seen (×400). (E) UR 0-7 kidney section showing also several glomerular and tubular positive TGF-β1 mRNA cellular staining (×200.) (F) Kidney from the UR 0-E group showing focal tubular TGF-β1 mRNA expression. Glomerular TGF-β1 mRNA staining is weak and scarce in the long-term treated groups (×200).
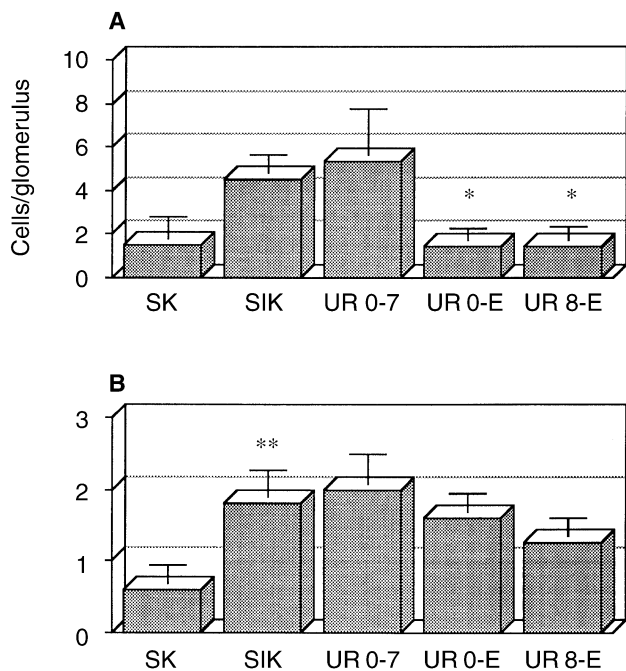
Fig. 4. Effect of UR-12670 on glomerular (A) and tubular (B) TGF-β1 mRNA expression. Five randomly selected kidney samples per group were analyzed. (A) In both UR-12670 long-term treated groups (UR 0-E and UR 8-E), glomerular cell TGF-β1 mRNA expression was significantly lower (*P < 0.03) than in the SIK group and the short-term treated group (UR 0-7) and was similar to the SK group (P = 0.9). (B) Tubular TGF-β1 mRNA expression was higher in the SIK group than in the SK group (**P = 0.03) and was similar to that in all UR-12670 treated groups. No differences were observed between UR 0-7, UR 0-E, and UR 8-E groups.

morphological benefits was analyzed by following animals for 52 weeks. Notably, short treatment with UR-12670 delayed and reduced proteinuria and glomerulosclerosis, similarly to what occurred when prolonged long-term treatment was used; however, these UR 0-7 treated ischemic rats disclosed late renal failure and severe chronic tubulointerstitial lesions. These findings suggest that protection of post-ischemic acute renal failure by UR-12670 only partially hindered the progression toward chronic nephropathy in this model. Other authors, treating post-ischemic acute renal failure either by a soluble form of P-selectin glycoprotein ligand [26] or by blocking the B7 costimulatory pathway with CTLA4Ig [27], found more evident long-term protection against chronic renal injury. A possible explanation may be that, in contrast to P-selectin glycoprotein ligand and CTLA4Ig treatment, which fully protected kidneys from organ dysfunction and leukocyte infiltration after ischemia/reperfusion injury, UR-12670–treated kidneys were only partially protected possibly because of the more prolonged warm ischemia we used. Moreover, ischemic untreated rats (SIK) developed chronic renal failure and achieved a fivefold higher proteinuria than rats in the previously

mentioned studies, in which 45 minutes of renal ischemia were employed [26, 27]. Thus, treating only the initial ischemia/reperfusion injury with UR-12670 does not seem to be sufficient to completely prevent the progression to chronic organ dysfunction, especially after a severe renal ischemic injury.

We designed two other groups in order to ascertain whether PAF is involved in the progression to chronic renal damage after ischemia/reperfusion injury in uninephrectomized rats. According to the renal protective effect of UR-12670, we can assume that acute renal lesions secondary to ischemia/reperfusion injury could probably be higher in the UR 8-E group than in the UR 0-E group. However, the survivors from both long-term UR-12670–treated groups showed similar attenuation of chronic renal dysfunction and renal lesions. These findings suggest that after ischemia/reperfusion injury and apart from the severity of acute renal failure, long-term blockade of PAF receptor interferes with the mechanisms involved in the progression of chronic nephropathy. This is clearly supported by the lower proteinuria, the absence of renal failure, and the low glomerulosclerosis, tubulointerstitial chronic damage, and vascular myointimal hyperplasia in long-term UR-12670–treated animals in comparison to the nontreated ischemic rats.

Platelet-activating factor has classically been involved as a key mediator in both the vasomotor and the inflammatory responses occurring early in postrenal ischemia [10, 11, 28, 29]. Our results suggest that PAF could also be considered as an important mediator of renal tissue matrix remodeling following ischemia in nephron mass ablation. The pathogenic mechanisms that lead to chronic organ dysfunction in this situation remain partially unknown, although ischemic organs are progressively infiltrated by T cells and macrophages, mainly located in glomeruli and around vessels [5, 22, 23]. The presence of these cell populations is associated with the release of biological mediators, such as interleukin-1, tumor necrosis factor-α, TGF-β1, endothelin-1, and the induction of nitric oxide synthase. Cells, cytokines, and chemoattractants, coupled with adhesion molecules, initiate a cascade that may ultimately stimulate proliferation of arterial smooth muscle cells, glomerular mesangial cells, and fibroblasts [5, 30]. In various models of chronic nephropathy, it has been demonstrated that infiltrating T cells and macrophages [31] as well as the intrinsic glomerular cells are sources of PAF [13, 14, 31, 32]. In addition, several mediators, including endothelin-1, induce the release of PAF by mesangial cells [33].

Recent studies have shown that TGF-β1 is a key fibrogenic cytokine involved in the pathogenesis of chronic nephropathy after ischemia and renal mass reduction [5, 27]. In addition, in experimental models of chronic renal injury, an overproduction of TGF-β1 has been linked to the development of glomerulosclerosis [34, 35].

Our results extend these observations because the ischemic kidneys showed a higher degree of TGF-β1 mRNA expression, both in glomerular and tubular cells, than nonischemic kidneys. In cultured mesangial cells, endogenous TGF-β1 has been identified as an important mediator of the stimulatory effect of PAF on matrix protein synthesis [13], suggesting that PAF contributes to the accumulation of mesangial matrix that occurs during glomerulosclerosis. According to these *in vitro* observations, in long-term UR-12670–treated kidneys, we found a clear reduction in the glomerulosclerosis as well as in the number of mRNA TGF-β1-positive glomerular cells down to the level disclosed in the nonischemic uninephrectomized rats. Our findings suggest that the contribution of PAF to glomerulosclerosis was mediated, at least in part, by the induction of TGF-β1 in glomerular cells. On the other hand, our results show that there were no significant differences in glomerulosclerosis between ischemic-treated UR 0-7 group and both long-term UR-12670 ischemic-treated groups. However, in contrast to long-term–treated groups, the UR 0-7 group displayed a significantly higher global and segmental glomerulosclerosis than nonischemic uninephrectomized rats. Nevertheless, there were apparently discordant results between the high glomerular TGF-β1 gene expression and moderate glomerulosclerosis in the ischemic treated UR 0-7 group. This might be partially explained by the normal serum cholesterol levels observed in these animals in comparison to the hypercholesterolemia in untreated ischemic rats, which, as we have shown, was clearly associated with the degree of proteinuria. It is well known that hypercholesterolemia can induce or aggravate preexisting glomerulosclerosis and artheriosclerosis by a mechanism involving platelet-derived growth factor-AB (PDGF-AB) [36, 37]. Thus, the absence of hypercholesterolemia in the short- and both long-term UR-12670–treated groups may also be important in the attenuation of glomerulosclerosis and vascular lesions. In contrast to glomerular TGF-β1 gene expression, tubular TGF-β1 in UR-12670 treated rats was similar to that in ischemic untreated rats, suggesting that the beneficial effect on tubular atrophy and interstitial fibrosis in long-term UR-12670–treated rats may be mediated by factors other than TGF-β1. In fact, although PAF induces an increase of extracellular matrix synthesis in cultured cells and interstitial fibroblasts [14], whether or not PAF acts directly or through other mediators different from TGF-β1 remains unknown.

Other mechanisms may also explain the implication of PAF in our chronic model. PAF alters the physicochemical properties of the glomerular capillary wall both by a direct effect [38, 39] or through the action of inflammatory cells [40] to induce proteinuria. It is known that abnormal glomerular permeability to proteins causes proximal tubular cell dysfunction by enhancing the secretion of extracellular matrix components by tubular cells and up-regulating inflammatory and vasoactive genes such as monocyte chemoattractant protein-1 (MCP-1), RANTES, and endothelin-1 (ET-1) [41, 42]. Moreover, recently it has been reported that proteinuria is the best predictor of progression toward chronic renal failure in nondiabetic patients [43]. An enhanced glomerular PAF production has been documented in some proteinuric nephropathies, and in these studies PAF receptor antagonists had an antiproteinuric effect [40]. Therefore, the delayed and moderate proteinuria in UR-12670–treated rats seen in our model could be related either to the initial reduction of the extent of acute renal lesions caused by ischemia/reperfusion injury (UR 0-7 and UR 0-E groups) or to the described antiproteinuric effect of PAF receptor blockade in the long term administration (UR 0-E and UR 8-E groups).

In summary, our results provide evidence that in the model of warm renal ischemia and mass reduction, long-term administration of the PAF receptor antagonist UR-12670 may be useful for the prevention of late renal dysfunction and morphological lesions. Moreover, our data further strengthen the idea that PAF plays a role in the mechanisms of progression of chronic renal damage.

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