Regression of Renal Vascular and Glomerular Fibrosis: Role of Angiotensin II Receptor Antagonism and Matrix Metalloproteinases

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Abstract. Renal fibrosis is one of the major complications associated with the development of hypertension. The objective of the present study was to determine whether and by which mechanisms treatment with AT1 receptor antagonists makes possible the regression of renal vascular and glomerular fibrosis. Experiments were performed in the hypertensive model of nitric oxide (NO) deficiency in rats. After 4 wk of hypertension, mortality rates averaged 20%; the surviving animals displayed a decline of renal function (urine protein/creatinine, 1.89 ± 0.63 versus 0.24 ± 0.03 mg/mmol; creatininemia, 110 ± 14 versus 38 ± 2 mmol/L in hypertensive animals and control, respectively; P < 0.01) and an exaggerated gene and protein expression of TGF-β, collagen I, and collagen IV (P < 0.001) within the renal vasculature associated with the development of glomerulosclerosis (sclerotic index, 2.26 ± 0.29 versus 0.12 ± 0.04; P < 0.001). In addition, activities of matrix metalloproteinases 2 and 9 were increased twofold in renal vessels and glomeruli (P < 0.01).

Afterwards, losartan, an antagonist of angiotensin receptor type I, or hydralazine were administered in subgroups of hypertensive animals. After 1 wk of angiotensin II antagonism, collagen I, or hydralazine were administered in subgroups of hypertensive animals. After 1 wk of AT1 receptor antagonism, collagen I, or hydralazine were administered in subgroups of hypertensive animals. After 1 wk of losartan, the renal functional and histologic parameters were completely normal, whereas they remained damaged in the hypertensive animals in which the mortality rate reached 85%. These data suggest that the progression of renal vascular fibrosis is a reversible process, at least in the NO deficiency model. The mechanism of the regression appears to be dual: inhibition of collagen synthesis due to AT1 receptor antagonism and activation of metalloproteinases that is probably associated with the degree of fibrosis independently of AT1 blockade.

Development of renal sclerotic lesions is one of the most common complications of hypertension. This pathophysiologic process is associated with changes in the structure of renal vasculature due to abnormal accumulation of extracellular matrix (particularly collagen types I, III, and IV) in renal resistance vessels, glomeruli, and interstitium (1). Although recent data obtained in the cardiac tissue indicate that the development of cardiac fibrosis is a reversible process under certain circumstances and treatments (blockade of angiotensin-converting enzyme [ACE] or antagonism of AT1 receptor) (2), very little is known about the capability of the renal vasculature to recover after the development of fibrotic injury.

In previous studies, we investigated the role of angiotensin II (AngII) in the mechanisms leading to the development of renal vascular and glomerular fibrosis using a strain of transgenic mice that express the luciferase reporter gene under the control of the promotor of the α2 chain of collagen I gene (3). We concluded that AngII played an important role in the abnormal expression of collagen I gene within the renal vasculature in the hypertension induced by nitric oxide (NO) inhibition, because the administration of AT1 receptor antagonists in a preventive mode protected kidneys from the development of fibrosis. Moreover, we found that the fibrogenic effect of AngII was at least partly mediated by an endothelin-1 action on collagen I gene (3,4). However, the experimental design of our previous studies did not allow conclusions about another important question, that is whether or not AT1 receptor antagonism can delay the progression of the sclerotic lesions in the renal vasculature, or even make them regress, and if so, whether this regression is due to the inhibition of the AngII action on collagen I gene or whether additional mechanisms involving systems that degrade collagens are also involved. These hypotheses, of a possible curative efficiency of AT1 receptor antagonists and of the underlying mechanism(s) involved, were examined in the present study.

Materials and Methods

Experimental Protocol

NO synthesis was inhibited by administrating Nω-nitro-L-arginine methyl ester (L-NAME, 20 mg/kg per d) in male Sprague-Dawley rats...
(250 g). In this model, 4 to 6 wk of L-NAME treatment are enough for the development of renal vascular and glomerular fibrosis in rats. The renal lesions observed during chronic inhibition of NO are glomerulosclerosis, glomerular ischemia, glomerular segmental necrosis, microvascular lesions, and interstitial expansion (5).

The experimental groups used were as follows: L-NAME 4 wk, animals were sacrificed (n = 20) after 4 wk of L-NAME treatment; L-NAME 8 wk, animals were sacrificed (n = 20) after 8 wk of L-NAME treatment; L-NAME 4 wk + LN+los 1 wk, animals were treated for 4 wk with L-NAME and then losartan was co-administered with L-NAME for an additional period of 1 wk (n = 24); L-NAME 4 wk + LN+hydr 1 wk, animals were treated for 4 wk with L-NAME and then hydralazine was co-administered with L-NAME for an additional period of 4 wk (n = 24); L-NAME 4 wk + LN+los 4 wk, animals were treated for 4 wk with L-NAME and then losartan was co-administered with L-NAME for an additional period of 1 wk (n = 8). The number corresponds to the animals used in the end of each experimental condition. Because the mortality rates differed between groups, the initial number of rats in each group varied accordingly. In parallel, two vehicle-receiving controls were used; one group was sacrificed at 4 wk (n = 20) and the other at 8 wk (n = 20). The vehicle groups gave similar results to all measured parameters; therefore, the 8-wk control data were presented in the figures of results. The doses of the drugs were based on preliminary experiments and previously published studies.

Measurement of BP

Systolic BP was measured by the tail-cuff method as described previously (3) using a piezoelectric sensor (Sensonor 840 to 01) and a data acquisition system composed of a Power PC Macintosh 4400/200 computer and a MacLab/4 s 16-bit analogue to digital converter (ADInstruments). Pressure recording was analyzed using the Chart module of the MacLab software. To avoid variations in BP due to day cycle, all measurements were carried out between 9 and 11 a.m. Animals were accustomed for several days before measurements were made. Eight measurements from each rat were taken at 2-min intervals, and a mean value was determined.

Isolation of Afferent Arterioles and Glomeruli

The technique to isolate afferent arterioles and glomeruli from the rat kidney, similar to that previously described (6), was based on iron oxide infusion into the renal artery and separation of the glomeruli and vessels with the aid of a magnetic field followed by successive passages through variously sized sieves. Vascular preparations containing >90% of afferent arterioles or glomeruli were retained for measurements of metalloproteinases 2 and 9 activities.

Renal Histology

Kidneys from at least five rats from each group were immersed in Dubosq solution. After fixation, four to six cortical slices of each kidney were embedded in paraffin after conventional processing (alcohol dehydration), and 3-μm-thick sections were stained with Masson trichromic solution for staining of extracellular matrix proteins and with Sirius red for collagen-specific staining.

Morphologic Evaluation

Sections of kidneys were examined on a blinded basis for the level of glomerular sclerosis and microvascular injury using the 0 to 4+ injury scale, as described previously (3). Injury scale 0 means no exaggerated extracellular matrix deposition in glomeruli, and 1+, 2+, 3+, and 4+ correspond to 1 to 25%, 25 to 50%, 51 to 75%, and 76 to 100% of increased extracellular matrix deposition per glomeruli, respectively. Sclerotic indexes of individual sections were averaged to calculate a sclerotic index for each rat. At least 500 glomeruli were scored to estimate the sclerotic index of a rat. Tubulointerstitial injury (defined as focal tubular atrophy, tubular dilatation with cast formation, interstitial expansion with perivascular inflammation, or thickening of the tubular membrane), was scored on a semiquantitative scale 0 to 4 as described previously (7).

Immunohistochemistry for Collagen I and IV and TGF-β

Four-micrometer-thick cryostat sections of renal cortex immunostained with an anti-collagen type I (1 mg/ml; Santa Cruz Biotechnology), anti-collagen IV (1 mg/ml; Pockland), or anti-TGF-β (1 mg/ml; Santa Cruz Biotechnology) at 1:50 for collagen and 1:100 for TGF-β, followed by FITC-coupled anti-goat IgG (5 mg/ml; Cappel) or Texas Red anti-rabbit (2 mg/ml; Molecular Probes), and washed with PBS/0.1% Tween. Photomicrographs were obtained with an Axioptophot Zeiss photomicroscope equipped with epifluorescence illumination.

Measurement of Urinary Protein Excretion

The day before sacrifice, animals were transferred in metabolic cages and urine samples were collected for a 24-h period. Urinary protein concentration was normalized to urinary creatinine concentration, and values were expressed as mg protein/μmol creatinine.

Measurement of Plasma Creatinine

Blood samples were withdrawn on the last day of the study, and creatinine was measured by the automated Jaffe method. Plasma creatinine concentrations were expressed as μmol/L.

Evaluation of Collagen I Synthesis in the Renal Tissue

Procollagen I carboxyterminal peptide (PICP) is freed during the extracellular processing of type I procollagen before the formation of collagen fibers. We have previously shown that increases of urinary excretion rates of PICP parallel the development of renal fibrosis, at least in the L-NAME model of hypertension (8). PICP was measured in urine by a RIA kit (Orion Diagnostica) and was normalized to urinary creatinine concentration.

Preparation of Complementary RNA Probes

Probes for collagen type I (chain α1), type IV (chain α1), and GAPDH were prepared from rat cDNA by RT-PCR using specific primers as previously published (8). The primers were as follows: for collagen I chain 1α, upstream: TGCTGCTTTTCTGTTCCTT, downstream: AAGGTGCTGGGTAGGGAAGT; for collagen IV chain 1α, upstream: TCGGCATTCTCTGCTT, downstream: TCTCGCTTTCTCCTATGGTG; for GAPDH, upstream: TGC-CACCTCAGAGACTTGG, downstream: GGATGCAGGGAT-GAGTTTCT. The corresponding expected fragment lengths were of 179, 185, and 84 bp for collagen I, collagen IV, and GAPDH, respectively. Synthesis of an antisense complementary RNA probe was carried out using an Ambion transcription kit.

Ribonuclease Protection Assay

RPA was performed as described previously (8). Briefly, renal cortical slices from control rats or rats treated with L-NAME for 4 wk, L-NAME for 4 wk plus L-NAME and losartan for 1 or 4 wk were
dissected, and RNA was extracted using Trizol reagent (Life Technologies BRL) according to a previously described protocol (8).

Real-Time Quantitative RT-PCR
Real-time quantitative RT-PCR was used to measure the steady-state levels of the mRNA of TGF-β, using acidic ribosomal phosphoprotein (P0) as standard. Total RNA was extracted using a High Pure RNA Isolation Kit according to the manufacturer’s instructions. The concentration and purity of RNA was determined by measuring the absorbance at 260 and 280 nm. For detection of P0, TGF-β, mRNA, 1 μg of total RNA were reverse transcribed into cDNA with 0.02 U/ml RT at 42°C for 50 min in standard buffer according to the manufacturer’s instructions. The following oligonucleotide primers were used: P0 sense primer 5’-CCCATCAGCACCAGGCC-3’, antisense primer 5’-CTCCAAGCAGATGAGCAGA-3’; TGF-β sense primer 5’-AGTGATCCAGCCAGCCAA-3’, antisense primer 5’-AGGAGGCAGCAGCATGTG-3’. DNA amplification was performed with 23 ng of cDNA using the BioRad technology; amplifications were performed with the ICyler- SYBR Green (Applied Biosystems). Reactions were cycled 32 times (denaturation at 95°C: TGF-β 15 s, P0 1 s; annealing: TGF-β 63°C, P0 58°C; extension at 72°C: TGF-β 10 s, P0 22 s; slopes were 20°C/Cs). Fluorescence was measured at the end of the extension phase. To confirm the specificity of the amplified products, melting curves were performed at the end of the amplification. For quantification, cDNA standards were used as standard in a tenfold serial dilution after determination of the concentration by absorbance at 260 nm.

Gelatinase Activity in Renal Cortex by In Situ Zymography
Renal cortical slices were excised, frozen in liquid nitrogen, and mounted with Tissue-Tek OCT compound (Sakura) for in situ zymography following a previously described protocol (9). Gelatinase activity was detected using D19 (Kodak) as emulsifier and Unifix (Kodak) for the fixation. Photomicrographs were obtained with an Axiohot Zeiss photomicroscope and digitalized using Thunder software.

Estimation of MMP2 and MMP9 Activities in Isolated Afferent Arterioles and Glomeruli
The enzymatic activities of metalloproteinases 2 and 9 (MMP2 and MMP9) were evaluated using gelatin zymography as described previously (10) using 15 μg of supernatants from isolated glomeruli or afferent arterioles and SDS-PAGE technique. The active forms of gelatinases (appeared as bands of 64 to 66 and 86 kD for MMP2 and MMP9, respectively) were digitalized and quantified using the Adobe Photoshop 6.0 and the NIH Image 1.61 software.

Statistical Analyses
Statistical analyses were performed using ANOVA followed by Protected Least Significance Difference Fisher test of the Statview software package. Results with \( P < 0.05 \) were considered statistically significant. All values are means ± SEM.

Results
Effect of L-NAME Treatment on Renal Function and Structure
Systolic BP rose after 4-wk of L-NAME treatment (182 ± 5 versus 109 ± 3 mmHg; \( P < 0.01 \); Figure 1). This increase was accompanied by deterioration of renal function as indicated by the abnormal increase of urinary protein excretion (\( P < 0.01 \)) and of plasma creatinine concentration (\( P < 0.001 \); Figure 1). At the same time, glomerulosclerosis and glomerular ischemia were clearly established as evidenced by Masson staining (Figure 2, B and C). Seventy-six percent of glomeruli displayed extracellular matrix scores from 2+ to 4+ (sclerotic index, 2.26 ± 0.39 versus 0.12 ± 0.04 in L-NAME 4-wk and control, respectively; \( P < 0.001 \); Figure 2, bottom); a similar pattern was observed with tubulointerstitial lesions (injury index, 0.87 ± 0.12 versus 0.08 ± 0.02 in L-NAME 4-wk and control, respectively; \( P < 0.001 \)). After 4-wk of L-NAME treatment, mortality rate averaged 20%.

Effect of L-NAME Treatment on Collagen Synthesis and Degradation
An important part of the exaggerated extracellular matrix formation at 4 wk was due to accumulation of collagens (Figure 3B); collagen I and IV mRNA expression and staining were increased within the renal vasculature (\( P < 0.001 \); Figure 4; Figure 5, B and F). The abnormal formation of collagens

Figure 1. Systolic BP (top), protein concentration in urine (middle), and creatinine concentration in plasma (bottom) from controls and rats treated for 4 wk or 8 wk with N\(^\circ\)-nitro-L-arginine methyl ester (L-NAME), or 4 wk with L-NAME followed by co-administration of L-NAME with losartan for 1 or 4 additional wk, or with hydralazine for 1 wk. Values are mean ± SEM of 20 to 24 animals/group in all conditions except the L-NAME + hydralazine group (\( n = 8 \)). * \( P < 0.01 \), ** \( P < 0.001 \) versus control; \( \# P < 0.01 \) versus L-NAME.
was accompanied by an increased urinary excretion of PICP ($P < 0.01$; Figure 6). In agreement with the literature (11), TGF-$\beta$ mRNA and protein expression were also increased in the glomeruli of $l$-NAME–treated animals (Figure 7, top and panel B). At the same time, enzymatic systems that degrade collag-
ens were also activated within the renal vasculature: as shown by in situ zymography, glomeruli from $l$-NAME rats (Figure 8B) displayed exaggerated gelatinase activity compared with

controls (Figure 8A). Quantitative estimation of MMP2 and MMP9 activities in freshly isolated glomeruli confirmed a twofold to threefold increase over control ($P < 0.01$; Figure 9). Similar observations were made in freshly isolated afferent arterioles ($P < 0.05$; Figure 10).

**Effects of AT1 Antagonism and Hydralazine on L-NAME–Treated Rats**

Treatment of the hypertensive animals with losartan markedly decreased BP. Thus, systolic pressure averaged $136 \pm 4$ mmHg after 1 wk and returned to control values after 4 wk of losartan (Figure 1). In addition to its anti-pressor effect, AT1 receptor antagonism markedly improved renal function as proteinuria and creatininemia were significantly decreased and reached control levels as early as 1 wk after treatment with losartan and remained normal until the end of the experiments (Figure 1). This improvement of renal function was accompanied by an amelioration of renal histology as evidenced by the reduced levels of extracellular matrix staining: 1 wk after losartan, glomerular fibrosis started to regress (Figure 2D) and almost completely disappeared after 4 wk (Figure 2F). Semiquantitative analysis of fibrosis indicated that $43\%$ of glomeruli exhibited a severe degree of glomerulosclerosis ($4+$) in the 8-wk L-NAME group (sclerotic index, $2.89 \pm 0.32$; Figure 2, bottom), whereas there were less than $6\%$ of glomeruli in class $4+$ after 1 wk and none after 4 wk of losartan (sclerotic index, $1.04 \pm 0.45$ or $0.09 \pm 0.01$, respectively; $P < 0.01$ versus L-NAME 8-wk; Figure 2, bottom). It is noteworthy that the degree of glomerulosclerosis early in the treatment with losartan (1 wk) is decreased even compared with the L-NAME 4-wk group ($P < 0.05$; Figure 2, bottom). Similarly, losartan treatment reduced tubulointerstitial injury as evidenced by the decrease of sclerotic index ($0.34 \pm 0.05$ and $0.10 \pm 0.02$ after 1 wk and 4 wk, respectively, $P < 0.01$ versus L-NAME 4-wk), whereas the injury index increased to $1.78 \pm 0.29$ in the 8-wk L-NAME group. The mortality rate reached $85\%$ in the L-NAME 8-wk group (survival rate, 20 of 132), whereas all animals under AT1 antagonist survived until the end of the protocol ($P < 0.001$). Losartan administration to control animals had no effect on the measured parameters of renal function and structure (Figures 1 and 2). In separate experiments, hydralazine was used to test whether another anti hypertensive agent could induce similar protection. After 1 wk of hydralazine treatment, systolic BP was reduced to similar levels compared with losartan (Figure 1, top); however, this decrease in systolic BP was not accompanied by a significant improvement of renal function (Figure 1, middle and bottom). The lack of protection was confirmed by the mortality rates; eight of ten animals were alive at 1 wk, and this number dropped to two surviving animals after 4 wk of L-NAME + hydralazine treatment.

**Effects of AT1 Receptor Antagonism and Hydralazine on TGF-β Expression, Collagen Synthesis, and Collagen Degradation**

Treatment of the hypertensive animals with losartan for 1 wk decreased the mRNA expression and protein levels of collagen
I and IV \( (P < 0.001; \text{Figures 4 and 5, C and G}) \); similar to collagen I expression, losartan normalized the exaggerated urinary excretion of PICP at 1 wk \( (P < 0.01; \text{Figure 6}) \). TGF-β expression levels were also decreased in glomeruli after AT1 receptor antagonism, but not by hydralazine (Figure 7, top and panels D and F). In contrast, the activities of MMP remained increased in glomeruli, as evidenced by \textit{in situ} zymography (Figure 8C). This observation was confirmed by the measurements of gelatinolytic activity in supernatants of glomeruli and afferent arterioles (Figures 9 and 10, respectively).

After 4 wk of losartan treatment, MMP activities were almost normalized in glomeruli (Figures 8E and 9) and afferent arterioles (Figure 10). At this time, no difference in the Sirius red or specific collagen I and IV staining was observed between the losartan-treated and control groups (Figures 3 to 6). In sharp contrast, the L-NAME 8-wk group exhibited abnormal collagen accumulation in the renal cortical tissue (Figure 3D), increased collagen I and IV staining (to levels similar with that observed with the L-NAME 4-wk group, not shown), increased urinary excretion rates of PICP (Figure 6), and exaggerated glomerular MMP activities (Figure 8 to 10).

**Discussion**

We have investigated the hypothesis that the development of renal vascular fibrosis is a reversible process, and we have looked at the mechanisms participating in this reversibility. An important novel finding is that fibrotic kidneys recovered completely, and the severely damaged renal morphology and function were restored to normal levels during therapy with an AT1 receptor antagonist. To our knowledge, this is among the first studies reporting an almost complete regression of renal vascular fibrosis. In addition, we provide some clues about the mechanism(s) involved in this regression. It appears that the recovery of renal structure resulted from a combination of two distinct mechanisms: inhibition of collagen synthesis (due to the blockade of AngII action) associated with increased metalloproteinase activity.

Several studies have demonstrated the involvement of the renin-angiotensin system in the profibrotic mechanisms and the efficacy of AngII blockers to prevent the development of renal, vascular, and cardiac fibrosis (12–15). Less known is the efficiency of AngII receptor antagonists as drugs promoting the regression of renal vascular fibrosis. This issue was one of the major goals of the present study. We found that blockade of the renin-angiotensin system after the formation of renal sclerotic lesions decreased the abnormal presence of extracellular matrix within the renal vasculature, and improved two parameters related to the glomerular function, proteinuria, and creatininemia. Interestingly, AT1 antagonism provided a considerably better protection than hydralazine for a similar anti-
hypertensive effect. This is in agreement with the literature: hydralazine administered in a preventive way (from the beginning of L-NAME treatment), failed to prevent the degradation of the renal function and structure in the L-NAME model, despite its effectiveness in normalizing BP (11). These results suggest that the mechanism(s) regulating renal remodeling are not dependent exclusively on systemic hemodynamics. In accordance with this notion, we have observed that angiotensin or endothelin receptor blockade canceled the activation of collagen I gene promoter in the renal vasculature and prevented the development of fibrosis independently of the BP levels (3,4).

Four weeks after losartan, in addition to normal proteinuria and creatininemia, no difference in morphologic and histologic markers could be observed between losartan-treated and control animals. This contrasted with the untreated group where the survivors displayed severe renal lesions. The major difference between untreated groups at 4 and 8 wk was that the mortality rose from 20% to 85%. The measurements and analysis of all parameters were performed in the surviving animals at the end of the treatments; therefore, there was a kind of "selection;" it is thus not surprising that the data of the group L-NAME 8 wk were similar to L-NAME 4 wk, because they concerned the best-preserved animals. The causes of mortality during long-term inhibition of NO are multiple and include cardiac failure and central nervous system damage, in addition to chronic renal failure (5).

The time course of the progression and regression of renal vascular fibrosis appeared to be a rapid phenomenon (both equal to 4 wk in our model), contrary to the notion that fibrosis is a pathology with a much slower pace (years in humans). Other investigators have also observed development of sclerotic lesions in rat kidneys after 4 to 6 wk of L-NAME treatment (15,16). Similarly, rapid development of renal sclerotic lesions has been reported in two other experimental models in the rat: radiation injury (glomerulosclerosis) and 5/6 nephrectomy (interstitial fibrosis) (17,18). It is possible that the collagen turnover is a species-dependent process. In this regard, we have observed that the same experimental model (L-NAME) of fibrosis displayed different kinetics in two different species: rats developed renal vascular fibrosis in a faster pace than mice (4 versus 10 to 14 wk), although the fibrogenic mechanism(s) were similar (AngII-dependent) (3,4,8).

The improvement in renal function and the restoration of renal vascular morphology during AT1 receptor antagonism could be due to a decreased synthesis and/or increased degradation of the components of extracellular matrix. In previous studies, we have established that AngII induces collagen I gene expression and renal fibrosis through the action of several other factors and pathways such as endothelin, TGF-β, MAP/ERK, and others.

**Figure 5.** Representative examples of immunostaining with collagen I (A through D) or collagen IV (E through H) antibodies in control (A and E) or rats treated either with L-NAME for 4 wk (B and F) or for 4 wk with L-NAME followed by L-NAME + losartan for 1 additional wk (C and G) or 4 additional wk (D and H). Note that the exaggerated collagen I and IV staining during inhibition of NO (B and F) was decreased after 1 wk (C and G) and disappeared after 4 wk of treatment with the AT1 antagonist (D and H). G, glomerulus, AR, arteriole. Scale bar, 10 μm.
In agreement with this notion, TGF-β1 expression in glomeruli was increased after 4 and 8 wk of L-NAME treatment, whereas losartan co-administration significantly decreased (1 wk) and completely normalized (4 wk) the exaggerated expression of TGF-β1. Other investigators have also observed that the locally activated renin-angiotensin system in connection with the increased TGF-β1 expression is a major pathogenic factor of renal injury in the L-NAME model (11). The novel finding of the present study that AT1 receptor antagonism inhibited the abnormal activation of collagen gene extends the previous findings and implies that an important step participating in the regression of renal vascular fibrosis is the inhibition of de novo synthesis of extracellular matrix proteins.

The above consideration leaves open the question of whether the regression of renal vascular fibrosis was solely due to the inhibition of AngII action on collagen synthesis or whether enzymes that degrade collagens such as matrix MMP have also participated. MMP expression is increased in a variety of human renal diseases and in animal models of nephropathies such as membranous nephropathy, polycystic kidney disease, and renal fibrosis (20). It is not clear however, whether the activation of these proteases promote or protect the kidney from injury. MMP9−/− mice developed a more severe form of anti-GBM nephritis than their wild-type mates, suggesting a protective action of MMP9 against crescentic proliferative glomerulonephritis (21). Studies in diabetic fibrotic nephropathy attributed the development of glomerulosclerosis to a decreased action of MMP due to an increased expression of their endogenous inhibitors TIMP (22,23). An early and sustained increase was observed in mRNA levels for collagens I, II, and IV, MMP1 and MMP2, and TIMP-1 in the model of subtotal nephrectomy (24). MMP1 and MMP2 activities were also increased, but proteolytic activities in remnant kidneys were reduced, an effect attributed to TIMP-1 action. Nevertheless, the development of interstitial fibrosis during obstructive nephropathy was not affected in TIMP-1 genetically deficient mice (25).

These apparent discrepancies could be explained by differences in the mechanisms leading to the development of renal fibrosis in various experimental models. In our model, the results obtained with in situ zymography show that MMP were activated locally in the renal cortical tissue during NO inhibition. It is possible that AngII was among the initiating factors of MMP activation through a TGF-β-dependent mechanism. In agreement to this notion, TGF-β administration increased MMP2 and MMP9 production in an in vitro model of tubular epithelial cells mimicking in vivo conditions (26); in addition, transgenic mice overexpressing TGF-β displayed increased glomerular levels of collagen I and III expression and elevated renal MMP-2 activity and developed glomerulosclerosis (27). We propose that the activation of MMP serves as a counterbalancing mechanism against the profibrogenic action of AngII and TGF-β. Once fibrosis is installed, the activation of MMP

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**Figure 6.** Urinary excretion rate of the pro-collagen I fragment P1CP in controls and rats treated for 4 wk or 8 wk with L-NAME, or 4 wk with L-NAME followed by co-administration of L-NAME with losartan for 1 or 4 additional wk. Values are mean ± SEM of 20 animals/group. * P < 0.01 versus control; # P < 0.01 versus L-NAME.
Figure 7. (Top) Real-time RT-PCR estimates of TGF-β expression in glomeruli from controls and rats treated for 4 wk or 8 wk with L-NAME, or 4 wk with L-NAME followed by co-administration of L-NAME with losartan for 1 or 4 additional wk. Values are mean ± SEM of six experiments (two rats/group per experiment). * P < 0.01 versus control; # P < 0.01 versus L-NAME. (Bottom) Representative examples of TGF-β expression in controls (A) and rats treated with L-NAME for 4 wk (B), 8 wk (C), or for 4 wk with L-NAME followed by co-administration of L-NAME with either losartan for 1 additional wk (D) or 4 additional wk (E) or hydralazine for 1 additional wk (F). Note that the intense staining of TGF-β during inhibition of NO (B and C) disappeared after losartan, but not hydralazine, administration (D through F).
becomes dependent of the presence of collagen, as several recent studies suggest (28,29). One week after losartan co-administration, collagen presence continued to be above normal levels and MMP2 and MMP9 remained activated. During this period, however, the stimulatory action of AngII and TGF-β on collagen synthesis was inhibited, shifting the balance toward collagen degradation. When collagen expression is normalized, 4 wk after losartan, MMP2 and MMP9 activities also returned to normal levels. The above-proposed explanation, however, is based on descriptive data regarding MMP activation. Additional studies with specific inhibition (pharmacologic or genetic) of MMP activation and/or expression are necessary to complete and confirm our hypothesis.

The notion that the formation of pathologic extracellular matrix around vessels is a dynamic process that can be reversed and that an efficient antihypertensive treatment should also address the issue of structural remodeling has recently emerged (30). This notion is based on data obtained in the cardiac tissue using mainly blockers of the renin-angiotensin system (31,32). Contrary to the heart, few data are available about the regression of fibrosis in the kidney. In the L-NAME model of hypertension in rats, blockers of AngII activity preserved kidney function and morphology in addition to normalizing systolic pressure (15,16). Similarly, blockade of the renin-angiotensin system improved the age-related decline of renal function (33–35). In these studies, however, antihypertensive treatments were introduced with little or mild degree of fibrosis within the kidney. In our protocol, the treatment started when the renal vasculature was severely fibrotic and the AT1 antagonist not only stopped the decline of animals’ health but completely restored renal function and structure to normal levels. Nevertheless, the issue remains open whether similar mechanisms apply to all stages of renal glomerular fibrosis. It is possible that the systems controlling the newly and the advanced end-stage fibrosis differ and thus the degree of reversibility and the therapies required may vary accordingly. Future studies addressing these issues will add valuable information about how to deal with the remodeling of the renal vasculature and the regression of renal fibrosis in the different stages of this physiopathologic process. Importantly, in support of the notion that renal fibrosis can also regress in human diseases, reversal of the lesions of diabetic nephropathy and improvement of renal function were recently observed in pancreas graft recipients, 10 yr after normalization of glycemia (36).

In conclusion, we investigated mechanisms of regression of renal vascular and glomerular fibrosis during hypertension induced by the prolonged deficiency of NO. Our data indicate that treatment with AngII receptor antagonist leads to the regression of renal lesions and improves the renal structure and function to almost completely normal levels, at least in this hypertensive model. This observation offers new insights in the
Figure 9. (Top) Representative display of MMP2 and MMP9 activities in supernatants of freshly isolated glomeruli from controls (lane 1) or rats treated either with L-NAME for 4 or 8 wk (lanes 2 and 4), or with L-NAME for 4 wk followed by L-NAME + losartan for 1 or 4 additional wk (lanes 3 and 5); lane 6: treatment with losartan alone. (Bottom) Effect of AT1 antagonist treatment on MMP2 and MMP9 activity in glomeruli of rats during inhibition of NO synthesis. Results are expressed as arbitrary units of optical densities of gelatinase activity. Values are mean ± SEM of four experiments (three to four rats/group per experiment). * P < 0.01 versus control; # P < 0.01 versus L-NAME.

Figure 10. (Top) Representative display of MMP2 and MMP9 activities in supernatants of freshly isolated afferent arterioles from controls (lane 1) or rats treated either with L-NAME for 4 or 8 wk (lanes 2 and 4), or with L-NAME for 4 wk followed by L-NAME + losartan for 1 or 4 additional wk (lanes 3 and 5); lane 6: treatment with losartan alone. (Bottom) Average values of MMP2 and MMP9 activity in afferent arterioles of rats during inhibition of NO synthesis. Results are expressed as arbitrary units of optical densities of gelatinase activity. Values are mean ± SEM of four experiments (three to four rats/group per experiment). * P < 0.05 versus control; # P < 0.05 versus L-NAME.
physiopathologic process of progression-regression of renal vascular fibrosis and can have important implications in the treatment of nephroangiosclerosis and glomerulosclerosis in human essential hypertension.

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