Regression of Renal Vascular Fibrosis by Endothelin Receptor Antagonism

Jean-Jacques Boffa, Pierre-Louis Tharaux, Jean-Claude Dussaule, Christos Chatziantoniou

Abstract—In previous studies, we have observed that endothelin participates in the progression of renal vascular and glomerular fibrosis during hypertension by activating collagen I gene synthesis. The present study investigated whether administration of endothelin receptor antagonists leads to the regression of renal sclerotic lesions. Experiments were performed in transgenic mice harboring the luciferase gene under the control of the collagen I-α2 chain promoter. Hypertension was induced by long-term inhibition of nitric oxide synthesis by Nω-nitro-L-arginine methyl ester (L-NAME); systolic pressure gradually increased, reaching a plateau of 165 mm Hg after 10 weeks of hypertensive treatment. At the same time, collagen I gene expression was increased 2- to 5-fold compared with control animals in afferent arterioles and glomeruli, respectively (P < 0.01). This increase was accompanied by the appearance of sclerotic lesions within the renal vasculature. When renal vascular lesions had been established (20 weeks of L-NAME), animals were divided into 2 subgroups: the one continued to receive L-NAME, whereas in the other, bosentan, a dual endothelin antagonist, was coadministered with L-NAME for an additional period of 10 weeks. Bosentan coadministration did not alter the increased systolic pressure at 30 weeks; in contrast, collagen I gene activity returned almost to control levels in renal vessels and glomeruli. In this subgroup of animals, renal vascular lesions (collagen and/or extracellular matrix deposition) and mortality rates were substantially reduced compared with untreated mice. These data indicate that endothelin participates in the mechanism(s) of renal vascular fibrosis by activating collagen I gene. Treatment with an endothelin antagonist normalizes expression of collagen I gene and leads to the regression of renal vascular fibrosis and to the improvement of survival, thus providing a complementary curative approach against renal fibrotic complications associated with hypertension. (Hypertension. 2001;37[part 2]:490-496.)

Key Words: hypertension, essential ▪ nephrosclerosis ▪ collagen ▪ extracellular matrix ▪ endothelin

Development of renal sclerotic lesions is one of the most common complications of hypertension.1 This pathophysiological process is associated with changes in the structure of renal vasculature caused by abnormal accumulation of extracellular matrix (mainly collagen type I) in renal resistance vessels, glomeruli, and interstitium.2 Several recent studies indicated that endothelial vasoactive agents such as nitric oxide (NO) and endothelin could be involved in the development of renal fibrosis. NO is an important inhibitor of vascular smooth muscle cell growth and extracellular matrix synthesis in vitro and in vivo,3,4 whereas chronic inhibition of NO synthesis is accompanied by renal vascular fibrosis.5,6 On the other hand, endothelin is a potent mitogenic agent in cultured vascular smooth muscle cells and mesangial cells,7,8 and endothelin antagonism is accompanied by prevention of vascular hypertrophy and fibrosis in several forms of experimental hypertension such as the DOCA-salt, angiotensin (Ang) II, or Nω-nitro-L-arginine methyl ester (L-NAME) models.9-11

In previous studies, we investigated the role of NO and endothelin in the initiation and development of renal vascular fibrosis using a new strain of transgenic mice.12 These mice express the luciferase reporter gene under the control of the promoter of the α2 chain of collagen I gene [procolla2(I)]13; the observation that luciferase and collagen I gene expression are closely correlated from the fetal development stage throughout the adult life under normal and/or pathological conditions12-14 makes this transgenic strain a model well adapted to studying the mechanisms whereby collagen I gene is activated, such as renal vascular and glomerular fibrosis. Using this model, we have found that the balance between NO and endothelin is a key factor for the control of collagen I gene expression in vivo: When endogenous NO is inhibited, endothelin synthesis is increased locally within renal vessels, and this increase plays a major role in activating collagen I gene expression, which ultimately leads to the development of renal vascular fibrosis.12

Little is known about the mechanisms maintaining renal vascular fibrosis. In the present studies, we pursued the interaction between endothelin, collagen I, and renal fibrosis by examining whether endothelin participates in the mechanism(s) controlling collagen I gene activation after sclerotic...
lesions had been established within the renal vasculature, and if so, whether the use of endothelin receptor antagonists could have curative implications by alleviating renal vascular fibrosis. Our findings imply an important role for endothelin in the control of extracellular matrix formation in the renal vasculature in the NO-deficiency model of hypertension. Endothelin receptor antagonism inhibits activation of collagen I gene and participates in the regression of vascular and glomerular fibrosis.

Methods

Animal Treatment
Male transgenic mice weighing 25 to 35 g (3 to 8 months old) at the time of the experiments were maintained on a normal salt diet. Animals had free access to chow and tap water. This transgenic line, named pGB 19.5/13.5, was generated in the laboratory of B. de Crombrugghe (University of Texas, Houston).13 These animals harbor a construction containing the sequences that this dose produced a gradual elevation of blood pressure estimated in pellets according to Bradford’s method. Results are expressed as luciferase Light Units per microgram of protein (LU/µg).

Measurement of Blood Pressure
Systolic blood pressure was measured by the tail-cuff method adapted to the mouse as previously described.12 Briefly, a piezoelectric sensor (Sensornor 840-01) connected to a carrier amplifier (Kent 2) was used to detect and convert heart pulses to electric signals. The outputs of the pressure transducer were interfaced to a data acquisition system composed of a Power PC Macintosh 4400/200 computer and a MacLab/4s 16-bit analog-to-digital converter (AD Instruments), allowing sampling at 40,000 samples per second. Pressure recording was analyzed with the chart module of the MacLab software.

Renal Histology
Kidneys from at least 4 mice from each group were immersed in Dubosq solution. After fixation, 2 to 3 cortical slices of each kidney were embedded in paraffin after conventional processing (alcohol dehydration), and 3-µm-thick sections were stained with Sirius red or Masson’s trichromic solution for staining of collagens or extracellular matrix proteins, respectively.

Morphological Evaluation
Sections of kidneys were examined on a blinded basis for the level of glomerular sclerosis and microvascular injury with the 0 to 4+ injury scale, as previously described.12 Injury scale 0 means no exaggerated extracellular matrix deposition in glomeruli; 1+, 2+, 3+, and 4+ correspond to 1% to 25%, 26% to 50%, 51% to 75%, and 76% to 100% of increased extracellular matrix deposition per glomeruli, respectively. The sclerotic index was considered to be equal to the sum 1×A+2×B+3×C+4×D, where A, B, C, and D represent the part of glomeruli belonging to classes 1 to 4, respectively. Twenty-five to 30 samples (at least 20 glomeruli per sample) were studied in each group.

Statistical Methods
Statistical analyses were performed with ANOVA followed by Fisher’s protected least-significance difference test of the Statview software package. Results with values of P<0.05 were considered statistically significant. All values are mean±SEM.

Results

Effect of L-NAME Treatment on procollα2(I) Gene Activation
In agreement with our previous results, systolic blood pressure rose after 6 weeks of L-NAME treatment (130±2 versus 118±2 mm Hg, P<0.01, Figure 1). Systolic pressure continued to rise with increasing duration of L-NAME treatment and reached a plateau around 160 to 170 mm Hg between 10

Luciferase Activity Assay
Luciferase activity was measured with a commercial reporter gene assay kit (Boehringer Mannheim). Tissues were frozen immediately after removal, and 500 µL of lysis buffer containing 0.1 mol/L KH2PO4/K2HPO4 (pH 7.8) and 1 mmol/L dl-dithiothreitol was added in each sample. Tissues were homogenized with a Polytron homogenizer, and cells were lysed by 3 freezing-defreezing cycles. Thereafter, samples were centrifuged at 12,000 g for 15 minutes, and luciferase activity was measured in 50 µL of supernatant with a Lumat LB 9507 luminometer (EG & Berthold). The protein content was estimated in pellets according to Bradford’s method. Results are expressed as luciferase Light Units per microgram of protein (LU/µg). In preliminary experiments, we verified that this luminometer has a linear range when assessing luciferase activity up to 107 LU.
and 30 weeks of treatment (Figure 1). Inhibition of NO synthesis increased luciferase activity in the renal vasculature before the onset of blood pressure increase. Isolated glomeruli displayed a 2-fold increase of luciferase activity after 4 weeks of L-NAME treatment (38 ± 3 versus 20 ± 2 LU/µg, \(P < 0.05\)). The L-NAME–induced activation of procollagen-I gene progressed with time (104 ± 9 LU/µg at 10 weeks, \(P < 0.01\)) and reached a 7-fold increase after 20 weeks (148 ± 11 LU/µg, \(P < 0.001\), Figure 2). Luciferase activity showed a similar pattern of increase in afferent arterioles and renal cortical slices (Figure 2); in afferent arterioles, for instance, luciferase activity started increasing after 4 weeks of L-NAME treatment (212 ± 12 versus 259 ± 10 LU/µg, for control and 4 weeks, respectively, \(P < 0.05\)) and reached a 2- to 3-fold increase at 20 weeks (516 ± 14 LU/µg, \(P < 0.01\), Figure 2). In agreement with our previous results, L-NAME treatment did not change procollagen-I gene expression in the two control (nonvascular but rich in collagen type I) tissues, tail, and skin (data not shown). In addition, as previously found, luciferase expression did not change with age (at least between 2 to 8 months) under control conditions in all tested tissues.12

Effect of L-NAME Treatment on Collagen and Extracellular Matrix Formation

Early in the development of hypertension (6 weeks), the renal cortical structure did not exhibit abnormal extracellular matrix accumulation as revealed by Sirius red and/or Masson’s staining (Figure 3, A and E). Renal vascular and glomerular fibrosis started to become evident at 10 weeks, and it was clearly established after 20 weeks of treatment (Figure 3, B and F). Semiquantitative evaluation of extracellular matrix formation after 20 weeks of L-NAME treatment confirmed renal injury in L-NAME–treated versus control mice (Figure 4). Almost all glomeruli displayed extracellular matrix scores from 2+ to 4+ (sclerotic index, 3.23 ± 0.03 versus 0.22 ± 0.02 in L-NAME 20 weeks and control, respectively, \(P < 0.001\)).

Effects of Endothelin Receptor Antagonism on the procollagen-I Gene Activation

The rationale of these experiments was based on our previous studies indicating that endothelin was involved into the mechanisms responsible for the activation of collagen I gene during the inhibition of NO synthesis.11,12 Thus, we investigated whether endothelin antagonism could delay the progression, or, even better, reverse renal vascular fibrosis. To this end, after 20 weeks of L-NAME treatment, bosentan, a mixed antagonist of endothelin receptors, was administered in vivo concomitant to L-NAME for an additional period of 10 weeks.

Endothelin receptor antagonism did not alter systolic blood pressure (167 ± 5 versus 162 ± 4 mm Hg, in mice treated with L-NAME for 30 weeks and L-NAME 20 weeks followed by L-NAME + bosentan, respectively, Figure 1). In agreement with our previous results,13 L-NAME treatment did not change procollagen-I gene expression in the two control (nonvascular but rich in collagen type I) tissues,
to control animals did not modify blood pressure or basal luciferase activity in any tested tissue (data not shown). In contrast, endothelin antagonism inhibited the L-NAME–induced activation of procolα2(I) gene in glomeruli at 30 weeks (194±13 versus 48±5 LU/μg, \( P < 0.01 \), for L-NAME–30 weeks and L-NAME+bosentan–treated animals, respectively, Figure 2, left). Similarly, bosentan reduced the increase in luciferase activity induced by L-NAME in afferent arterioles (539±29 versus 302±24 LU/μg, \( P < 0.01 \), Figure 2 middle) and renal cortex (468±32 versus 252±16 LU/μg, \( P < 0.01 \), Figure 2, right), implying a specific action of bosentan on renal vasculature independent of the increase of systolic blood pressure.

Effects of Endothelin Receptor Antagonism on the Extracellular Matrix Formation

Antagonism of endothelin receptors markedly protected kidneys from the L-NAME–induced fibrosis as evidenced by the reduced levels of collagen and extracellular matrix staining in L-NAME+bosentan compared with L-NAME 30 weeks...
group (Figure 3, C and D, G and H, respectively). Semiquantitative analysis of fibrosis indicated that 40% of glomeruli exhibited a severe degree of glomerular injury (4+) in the L-NAME group (sclerotic index, 3.27±0.06) (Figure 4), whereas there were <15% of glomeruli in class (4+) in the L-NAME+bosentan group (Figure 4; sclerotic index, 2.49±0.10; P<0.01). Interestingly, bosentan blunted the degree of glomerulosclerosis, even compared with L-NAME 20 weeks group (sclerotic index 3.23±0.03, P<0.01).

Effects of Endothelin Receptor Antagonism on Mortality Rate
This improvement of renal vascular histology during cotreatment with bosentan was accompanied by a net reduction of mortality rates. In the L-NAME group, some mice died after 24 weeks, and the mortality rate reached 36% (9 of 25) of animals by 30 weeks. In contrast, some mice died at 28 weeks in the L-NAME+bosentan group, and mortality rate was ≈6% at 30 weeks (1 of 16).

Discussion
In this study, we used a strain of transgenic mice harboring the luciferase reporter gene under the control of collagen I promoter to investigate the mechanisms of regression of renal vascular fibrosis. We could show a major role for endothelin in controlling collagen I gene activation in renal vessels during inhibition of NO synthesis. An important novel finding is that bosentan, an endothelin receptor antagonist, given in a curative way, inhibited the activation of procoIa2(I) and reduced the severity of renal lesions, although it did not reduce systolic blood pressure. This result implies that during chronic inhibition of NO synthesis (a) endothelin is involved in the progression of renal vascular fibrosis by acting on collagen I gene, and (b) administration of endothelin receptor antagonists could reverse this fibrotic process independent of systemic hemodynamics.

Several recent studies point to a major role for endothelin in mediating renal fibrosis. Antagonism of endothelin receptors delayed the evolution of renal failure and increased the survival rate in rats with renal mass reduction.15 Similarly, the use of an endothelin receptor antagonist improved renal structural damage and reduced extracellular matrix (including collagen I) formation in the model of murine lupus nephritis.16 Transgenic mice overexpressing human endothelin 1 gene developed glomerulosclerosis and interstitial fibrosis without change in arterial pressure, thus corroborating the hypothesis that the endothelin-mediated fibrogenic mechanisms are independent of systemic hemodynamics.17

In recent studies, we provided additional elements on the mechanisms by which endothelin participates in the development of renal vascular fibrosis. Our data pointed to a local action of endothelin in the renal vascular beds: mRNA expression and peptide content of endothelin in renal resistance vessels and endothelin urinary excretion rate were increased in rodents treated with L-NAME.11,12 We have also found that this local activation of endothelin occurred concomitantly with an increase in the promoter activation of collagen I in renal microvessels and glomeruli. Bosentan given in a preventive way abolished the exaggerated procoIa2(I) activity and mRNA expression and peptide synthesis of collagen I and markedly protected the renal vasculature from the development of fibrosis.11,12 Interestingly, these protective effects of endothelin antagonism occurred despite the persistent increase of systolic pressure in L-NAME–treated animals. In agreement with our studies, other investigators observed that selective blockade of ETα receptors prevented proteinuria and glomerular ischemia and blunted the degree of vascular and tubulointerstitial injuries during inhibition of NO synthesis without normalizing blood pressure.18

The above studies investigated mainly mechanisms leading to the development of renal vascular fibrosis, in which pharmacological agents were administered in a preventive fashion. Little is known regarding the involvement of endothelin in mechanisms maintaining renal vascular fibrosis and the efficiency of endothelin receptor antagonists as curative drugs against this disease. A major goal of the present study was to investigate these hypotheses. We observed that an endothelin receptor antagonist given 20 weeks after the beginning of hypertension markedly inhibited collagen I gene activation and that this inhibition was followed by a decrease of the abnormal collagen and extracellular matrix formation within the renal vasculature. Animals receiving bosentan displayed a less severe degree of glomerular lesions even compared with those at the beginning of the treatment (L-NAME 20 weeks, Figures 3 and 4), thus suggesting that
renal vascular fibrosis regressed during treatment with an endothelin receptor antagonist. The parameters of semiquantitative analysis led to the impression that renal fibrosis did not evolve (worsen) between 20 and 30 weeks (similarity of values for sclerotic index, percentage of injured glomeruli, Figure 4). However, we do not think that this was the case, because mice started to die after 24 weeks of L-NAME treatment, and mortality rate reached 35% by the 30th week. As a result, there was a kind of “selection,” and the data in Figures 3 and 4 may concern the best “preserved” animals in this group.

It would be interesting to investigate whether the bosentan-induced improvement of renal structure was accompanied by an improvement of renal function and whether the increase of survival rate could be attributed to the renal function. With measurements of classic parameters of renal function, we encountered some practical problems in mice because of the size of this animal and because control mice (at least in our strain) excrete proteins in urine to levels above the normal standards for other species. An alternative could be the use of rats as an animal model for these renal function studies. In an ongoing study performed in our laboratory, we have observed that, as with mice, endothelin antagonism induced a partial regression of renal vascular fibrosis in L-NAME–treated rats. This regression was associated with an improvement of renal function (proteinuria, creatininemia) and survival rates; interestingly, it appeared that at least a part of the decreased mortality rate was due to renal function amelioration (Boffa et al, unpublished observations).

The concept that an efficient treatment against hypertension and its complications should also address the issue of pathological structural remodeling (in addition to simply lowering blood pressure) has recently emerged. This concept is based on data obtained in the cardiac tissue with mainly blockers of the renin-angiotensin system. Long-term treatment with an ACE inhibitor produced regression of left ventricular hypertrophy and normalized blood pressure in young spontaneously hypertensive rats (SHR). Interestingly, when the same ACE inhibitor was given in a nonpressor dose, perivascular and interstitial fibrosis regressed and myocardial stiffness was normalized. A reduction of cardiac fibrosis with a concomitant increase of matrix metalloproteinase activity was also observed in old SHR after long-term antihypertensive treatment with an ACE inhibitor. In two recent studies, performed in a relatively limited number of hypertensive patients, ACE inhibition diminished the volume of perivascular collagen and improved cardiac function. Contrary to the heart, there is a paucity of data concerning mechanism(s) of fibrotic regression in the renal vasculature, another primary target of hypertensive diseases. Treatment with a calcium blocker and/or an ACE inhibitor improved renal hemodynamics and prevented nephrosclerosis in SHR treated with L-NAME. In the L-NAME model of hypertension in rats, inhibition or antagonism of Ang II preserved kidney function and morphology in addition to normalizing systolic pressure. However, the pharmacological treatment and the normalization of blood pressure started early, when the degree of sclerotic lesions was relatively low; ACE inhibition ameliorated parameters of renal function and histology compared with the nontreated animals in the end of experiments but not compared with the animals in the beginning of the treatment. Thus, these observations are more relevant to a protection against the progression rather than to a regression from an existing fibrosis. To our knowledge, the present study is among the first reporting an improvement of renal morphology after a long-term induction of the hypertensive pathology. In support of the “regression of renal fibrosis” hypothesis, reversal of the lesions of diabetic nephropathy was observed in patients 10 years after pancreas transplantation and induction of normoglycemia.

Although bosentan almost completely inhibited collagen I gene activation, it partially restored renal vascular histology. Animals treated with bosentan continued to display exaggerated extracellular matrix formation compared with age-matched control animals (Figure 3). A possible explanation is that the timing of observation (10 weeks of curative treatment) was not long enough to completely eliminate the already accumulated extracellular matrix. The turnover for the degradation of collagen I depends on species, tissue, and/or pathology. In the rat, for instance, where L-NAME–induced renal fibrosis occurs faster than in the mouse (hypertension and severity of renal lesions developed faster during L-NAME administration in rat than in mouse), curative treatment with an AT\_1 antagonist for 1 to 2 months can almost completely normalize renal morphology (Boffa et al, unpublished data). It is also possible that endothelin, being one of several mediators participating in the renal fibrotic process blockade of its action, does not inactivate other fibrogenic systems. For instance, transforming growth factor-β is one of the most potent signals for the induction of extracellular matrix synthesis and renal fibrosis and is considered to mediate a major part of the fibrotic action of angiotensin II. Mechanical strain is another signal that can activate synthesis of extracellular matrix proteins, such as fibronectin and collagens, in human vascular smooth muscle cells in vitro. It would be interesting to investigate in future studies whether there is an interaction between endothelin and transforming growth factor-β and to examine the role that could play the hypertension-induced increase of contractility in renal resistance vessels or the involvement of systems that degrade collagens (such as the family of matrix metalloproteinases) in the process of regression of renal fibrosis.

Conclusions

We investigated mechanisms of regression of renal vascular and glomerular fibrosis by using a new model of transgenic mouse harboring the luciferase reporter gene under the control of the collagen I promoter. Our data indicate that endothelin plays an important role in maintaining renal vascular fibrosis and that chronic inhibition of its action leads not only to the prevention as already reported but also to the regression of renal lesions independent of systemic hemodynamics. This observation can have important implications in the treatment of nephroangiosclerosis and glomerulosclerosis in human essential hypertension.
Acknowledgments
This work was supported by the “Institut National de la Santé et de la Recherche Médicale” and the “Faculté de Médecine St Antoine.” The authors thank Drs George Bou-Gharios, Jérôme Rossett, and Benoît de Crombrugghe (Department of Molecular Genetics, University of Texas, Houston) for providing the transgenic mice and Dr Martine Clozel (Actelion, Basel, Switzerland) for providing bosentan.

References
Regression of Renal Vascular Fibrosis by Endothelin Receptor Antagonism
Jean-Jacques Boffa, Pierre-Louis Tharaux, Jean-Claude Dussaule and Christos Chatziantoniou

Hypertension. 2001;37:490-496
doi: 10.1161/01.HYP.37.2.490
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/37/2/490

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/