Reversal of renal lesions following interruption of nitric oxide synthesis inhibition in transgenic mice

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Abstract

Background. Renal fibrosis, a common complication of hypertension and diabetes is considered as a non-curable disease and is characterized by the abnormal accumulation of collagen I within the kidney. Chronic inhibition of nitric oxide (NO) synthesis is a model of hypertension associated with the development of nephroangiosclerosis. The present study investigated whether halt of NO inhibition leads to the regression of renal sclerotic lesions.

Methods. The NO deficiency (N\textsubscript{G}-nitro-L-arginine methylester; L-NAME) model of hypertension was applied in transgenic mice harbouring the luciferase reporter gene under the control of the collagen I-\alpha\textsubscript{2} chain promoter.

Results. Systolic pressure gradually increased following the administration of L-NAME, and reached 160 mmHg after 8–10 weeks. Activation of collagen I gene within the renal vasculature preceded the blood pressure increase and was accompanied by the appearance of sclerotic glomeruli and tubulointerstitial infiltration. After renal lesions had been established (20 weeks), animals were divided in three subgroups for an additional experimental period of 10 weeks: first group continued to receive L-NAME, in the second, L-NAME administration was stopped to allow endogenous NO synthesis and in the third the removal of L-NAME was combined with endothelin receptor antagonism. Removal of L-NAME decreased, without normalizing, systolic pressure and collagen I gene activity; renal morphology was substantially improved, and tubulointerstitial infiltration disappeared. Combination of L-NAME removal with endothelin antagonism normalized collagen I gene expression and further improved renal morphology without further decreasing blood pressure.

Conclusion. Manipulating the balance between NO/vasoconstrictors in favour of NO could provide a curative approach against renal inflammatory and fibrotic complications associated to hypertension.

Keywords: nitric oxide synthesis; renal fibrosis; hypertension; collagen gene activation
had been established within the renal vasculature, and if this strategy could ultimately lead to the regression of renal vascular fibrosis. The findings indicate that NO inhibits activation of collagen I gene and, combined with endothelin receptor antagonism, can induce an almost complete regression of fibrosis and recovery of renal structure.

**Methods**

**Animal treatment**

Male transgenic mice weighing 25–35 g and aged 3 and 10 months at the beginning and the end of the experiments, respectively, 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 (Univ. of Texas, Houston, Tx) [4]. These animals harbour a construction containing the sequences −19.5 to −13.5 kb and −350 to +54 bp of the promoter of the α2 chain of mouse collagen type I gene linked to two reporter genes, the firefly luciferase and the *Escherichia coli* β-galactosidase. The choice of the mice was based on the previous studies showing that the expression pattern of the two reporter genes in embryos and adult animals closely correlates with cell and tissue distribution of collagen I and does not change between 3 and 10 months of age [1–4].

NO synthesis was inhibited by administrating N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME), a NO synthase inhibitor (20 mg/kg/day orally in the drinking water). We have previously found that this dose produced a gradual elevation of blood pressure accompanied by the appearance of glomerular and renal vascular lesions after 10–12 weeks of treatment [1]. After 20 weeks of L-NAME treatment (when lesions appeared to reach more than 80% of glomeruli), mice were divided into three subgroups: the first continued to receive L-NAME; in the second, L-NAME was removed from the drinking water to allow reactivation of endogenous NO synthesis; and in the third the L-NAME removal was accompanied by administration of bosentan (20 mg/kg/day), a dual endothelin receptor antagonist. Bosentan was first dissolved in a small volume of sodium bicarbonate and then added to the drinking water. A similar volume of sodium bicarbonate was used as placebo in the L-NAME removal group. These groups were observed for an additional period of 10 weeks and 20–25 mice were utilized per experimental condition.

In a separate group of 7-month-old animals a NO donor, molsidomine (1 mg/kg/day), was administered in addition to L-NAME treatment for a period of 8 weeks. In parallel, vehicle-receiving controls were sacrificed at 10-, 20- and 30- week intervals (n = 8 for each time). Since vehicle groups gave similar results to all measured parameters, the pooled data were presented in the figures of results. The doses of the drugs were based on preliminary experiments and previously published studies.

**Isolation of preglomerular vessels and glomeruli**

The technique to isolate preglomerular vessels and glomeruli from the transgenic mouse kidney was similar to that previously described [3]. In mice anesthetized with pentobarbital, a midline abdominal incision was made, and the abdominal aorta was cannulated (Surflo 24G catheter, Terumo) below the renal arteries. The aorta above the kidney was ligated, the left renal vein was cut, and kidneys were perfused with ice-cold isotonic saline solution until all blood had been removed. Thereafter, the kidneys were perfused with a magnetized iron oxide suspension (1% Fe\textsubscript{3}O\textsubscript{4} in isotonic saline). All subsequent steps of isolation were performed at 4°C. The kidneys were removed, decapsulated, and the cortex was dissected from the medulla. The cortical tissue was homogenized with a Polytron homogenizer, and the iron oxide-loaded tissues (renal vessels and glomeruli) were removed from the crude homogenate with the aid of a magnet. Preglomerular vessels were separated from larger vessels and glomeruli using repetitive passages through needles and sieves of decreasing diameter sizes (23–26 gauge and 75–25 μm, respectively). The microvessels were recovered from the top of the 50 μm sieve and the glomeruli from that of the 25 μm sieve. To determine if parts of preglomerular vessels stayed attached to glomeruli during the separation process, glomeruli were examined under light microscopy. Only a minor part of isolated glomeruli (~3%) had an attached portion of their afferent arteriole. Vascular preparations containing >90% of preglomerular vessels or glomeruli were retained for luciferase activity measurements. Kidneys from four mice were used to isolate preglomerular vessels and glomeruli for each experiment.

**Luciferase activity assay**

Luciferase activity was measured using a commercial reporter gene assay kit (Boehringer Mannheim, Germany). Tissues were frozen immediately after removal, and 500 μl of lysis buffer containing 0.1 M KH\textsubscript{2}PO\textsubscript{4}/K\textsubscript{2}HPO\textsubscript{4} (pH = 7.8) and 1 mM DL-dithiothreitol were added in each sample. Tissues were homogenized with a Polytron homogenizer, and cells were lysed using three freezing–defreezing cycles. Thereafter, samples were centrifuged at 12 000 × g for 15 min, and luciferase activity was measured in 50 μl of supernatant using a Lumat LB 9507 luminometer (EG & Berthold, France). The protein content was estimated in pellets according to Bradford’s method. Results are expressed as luciferase light units per μg of protein (LU/μg). In preliminary experiments we verified that this luminometer has a linear range when assessing luciferase activity up to 10\textsuperscript{7} LU.

**Measurement of blood pressure**

Systolic blood pressure was measured by the tail-cuff method adapted to the mouse as previously described [1–3]. Briefly, a piezoelectric sensor (Sensocon 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 (ADInstruments) allowing sampling at 40 000 samples/s. Pressure recording was analysed using the Chart module of the MacLab software.

To avoid variations in blood pressure due to day cycle, all measurements were carried out between 9 and 11 am. Animals were acclimatized for several days before measurements were made. Eight measurements from each mouse were taken at 2 min intervals, and a mean value was determined.
Renal histology

Kidneys from at least six mice from each group were immersed in Dubosq solution. After fixation and conventional processing, 2–3 cortical slices of each kidney were embedded in paraffin (alcohol dehydration), and 3 μm-thick sections were stained with Masson trichromic solution for staining of extracellular matrix.

Morphologic evaluation

Sections of kidneys were examined on a blinded basis for the level of glomerular sclerosis and microvascular injury using the 0–4+ injury scale, as previously described [1–3]. Injury scale 0 means no exaggerated extracellular matrix deposition in glomeruli, while 1+, 2+, 3+ and 4+ correspond to 1–25, 26–50, 51–75 and 76–100% of increased extracellular matrix deposition per glomeruli, respectively. Sclerotic indexes of individual sections were averaged to calculate a sclerotic index for each mouse. At least 200 glomeruli were scored to estimate the sclerotic index of an animal.

Statistical methods

Statistical analyses were performed using ANOVA followed by Protected Least Significance Difference Fisher’s 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 the collagen I gene activation and extracellular matrix formation

In agreement with the previous results, systolic blood pressure rose after 6 weeks of L-NAME treatment (125 ± 2 vs 115 ± 2 mmHg, P < 0.05). Systolic pressure continued to rise with increasing duration of L-NAME treatment and stabilized at 160 mmHg after 10 weeks of treatment (Figure 1). Luciferase activity was continuously increasing following NO inhibition in glomeruli and preglomerular vessels, and reached a several-fold increase over control values after 20 weeks (P < 0.001, Figure 2). In agreement with the previous results [1–3], L-NAME treatment did not change collagen I gene expression in the two control (non-vascular but rich in collagen type I) tissues, tail and skin (data not shown). Confirming the previous results [1–3], luciferase expression did not change with age under control conditions in any tested tissue, at least between 4–10 months of age.

Early in the development of hypertension (before 10 weeks), the renal cortical structure did not exhibit abnormal extracellular matrix accumulation as revealed by Masson’s staining (Figure 3A). Thereafter, the first sclerotic glomeruli appeared, and their number was substantially increased after 20 weeks of hypertension (Figure 3B). Peritubular inflammation was also evident in the same period (Figure 3B). Semi-quantitative evaluation of sclerotic glomeruli confirmed renal injury after 20 weeks of L-NAME treatment (glomerulosclerosis index of 3.24 ± 0.03 vs 0.23 ± 0.02 in L-NAME 20 weeks and control, respectively, P < 0.001, Figure 4).
Collagen I gene activation and extracellular matrix formation following L-NAME removal

To test whether renal fibrosis is a reversible process, L-NAME was removed from the drinking water after 20 weeks of hypertension in a subgroup of animals. It has been previously shown that this manipulation reactivates endogenous NO synthesis and is accompanied by a decrease in blood pressure [5]. In agreement with the previous observations, systolic pressure was reduced, but not normalized, after 10 weeks of L-NAME removal (125±3 mmHg, \(P < 0.01\) vs L-NAME 30 weeks; \(P < 0.05\) vs control Figure 1).

Fig. 3. Representative examples of extracellular matrix (Masson’s trichrome A–E) in transgenic controls (A) and mice treated with L-NAME for 20- (B), 30 weeks (C), 20 weeks with L-NAME followed by placebo for 10 additional weeks (D) or 20 weeks with L-NAME followed by bosentan for 10 additional weeks (E). Note the extracellular matrix accumulation and the peri-tubular cell infiltration during chronic inhibition of NO (B and C); glomerular lesions and tubular inflammation clearly regressed after 10 weeks of removal of L-NAME without or with bosentan (D–E).
L-NAME removal profoundly decreased collagen I gene expression in glomeruli (196±12 vs 53±9 LU/mg, P<0.01, for L-NAME 30 weeks and L-NAME-removed animals, respectively, Figure 2 upper panel); this decrease was less important in preglomerular vessels (539±20 vs 441±20 LU/mg, P<0.05, for L-NAME 30 weeks and L-NAME-removed animals, respectively, Figure 2 lower panel).

Withdrawal of L-NAME markedly protected kidneys from fibrosis as evidenced by the reduced levels of mesangial extracellular matrix staining in L-NAME-removed group compared to L-NAME 30 weeks group (Figure 3C vs D). Semi-quantitative analysis of glomerulosclerosis indicated that 50% of glomeruli exhibited a severe degree of glomerular injury (4+) in the L-NAME group (index of glomerulosclerosis 3.26±0.04) (Figure 4), whereas there was less than 10% of glomeruli in class (4+) after removal of L-NAME (Figure 4, index of glomerulosclerosis 2.27±0.06, P<0.01). This decrease was evident even compared to L-NAME 20 weeks group (3.24±0.03), suggesting a regression of glomerulosclerosis. This improvement of renal cortical structure was accompanied by the disappearance of infiltrating cells (Figure 3D).

**Effect of a NO donor on collagen I and extracellular matrix formation**

To verify that the inhibition of collagen I gene activation was linked to NO action, a subgroup of animals receiving L-NAME was treated with the NO donor molsidomine. Chronic administration of molsidomine prevented systolic pressure increase (118±3 mmHg) and collagen I gene activation in glomeruli (25±3 vs 102±11 LU/mg) and afferent arterioles (215±27 vs 437±20 LU/mg in L-NAME + molsidomine and L-NAME-treated mice, respectively Figure 5). This inhibition of collagen I gene activation was accompanied by a complete preservation of renal structure (data not shown).

**Effect of the association of L-NAME removal with endothelin receptor antagonism on collagen I and extracellular matrix formation**

The rationale of these experiments was based on the previous findings that endothelin was a key activator of collagen I gene during prolonged inhibition of NO synthesis [1–3]. Thus, we investigated whether the addition of endothelin antagonism could facilitate the recovery from fibrosis. To this end, after 20 weeks of L-NAME treatment, bosentan, a dual antagonist of endothelin receptors, was administered concomitant to L-NAME withdrawal for an additional period of 10 weeks.

The addition of endothelin receptor antagonist did not further reduce systolic blood pressure (128±3 mmHg, Figure 1), but it decreased further collagen I gene expression in glomeruli and preglomerular vessels (P<0.01 vs L-NAME-20 weeks + placebo 10 weeks, Figure 2). In both tissues, collagen I gene activity returned to basal values, implying a specific action of endothelin antagonism on collagen I independent of the variations of systolic blood pressure. In agreement with our previous results, bosentan administration did not modify blood pressure or basal control.
Luciferase activity in any tested tissue of control animals (data not shown).

In addition, the kidney structure was markedly restored in animals treated with endothelin receptor antagonist for 10 weeks (group: L-NAME-20 weeks + bosentan 10 weeks) compared to animals receiving L-NAME for -20 or -30 weeks (Figure 3E vs B and C). Index of glomerulosclerosis was reduced to 1.61 ± 0.10, a value lower than that of the L-NAME removal group (Figure 4). Again, this improvement was accompanied by an absence of infiltrating cells (Figure 3E).

Discussion

In the present study, we investigated the mechanisms of regression of renal vascular fibrosis. A major novel finding is that inhibition of collagen I synthesis and regression of renal fibrosis can be achieved after correction of the initiating cause of the pathology (inhibition of NO synthesis). The degree of the regression is even better when endothelin receptor antagonism is combined to L-NAME removal. This result implies that the balance between NO and endothelin plays a major role in the mechanisms controlling progression and regression of renal vascular and glomerular fibrosis and that modulation of this equilibrium could have important therapeutic effects against chronic renal failure, a disease considered incurable.

Endothelial dysfunction and the interaction between NO and endothelin are considered as key events for vascular remodeling. NO is an important inhibitor of vascular smooth muscle cell growth and extracellular matrix synthesis in vitro and in vivo [7], while chronic inhibition of NO synthesis is accompanied by renal vascular fibrosis [8,9]. On the other hand, endothelin is a potent mitogenic agent in cultured vascular smooth muscle and mesangial cells and endothelin antagonism is accompanied by prevention of vascular hypertrophy and fibrosis in several forms of experimental hypertension such as the DOCA-salt, angiotensin II or L-NAME models [6,10,11]. In addition, antagonism of endothelin receptors delayed the evolution of renal failure and increased the survival rate in rats with renal mass reduction and in the model of murine lupus nephritis [12,13].

The above studies established that inhibition of NO and/or activation of endothelin are mediators of the progression of renal vascular fibrosis. From a therapeutic point of view, however, it is more relevant to investigate whether reversal of renal lesions can be achieved. This was a major objective of the present study. We observed that stimulation of collagen I synthesis ceased with L-NAME removal and that morphology was improved compared to that observed at the moment of halt of L-NAME (Figure 3B vs D) implying a regression of renal fibrosis. This regression could be due to a direct action of NO against collagen I, as suggest experiments in which NO donors directly inhibited collagen I synthesis in vascular smooth muscle cells [14]. It is also possible that the recovery of renal structure could be a consequence of the anti-hypertensive action of NO, since the removal of L-NAME or the administration of molsidomine were accompanied by a substantial improvement of systolic blood pressure (Figures I and 5).

The addition of endothelin antagonist normalized collagen gene activity and further improved the degree of the recovery. Since these additional improvements occurred without any further decrease of blood pressure, they may have resulted from a direct inhibition of collagen I synthesis. In support of this hypothesis, we have previously found that endothelin induces collagen I gene activation through a mechanism involving transactivation of EGF receptor and the MAP/ERK pathway [1,6,15]. In agreement with our results, other investigators observed that selective blockade of ETA receptor prevented proteinuria and glomerular ischaemia and blunted the degree of vascular and tubulointerstitial injuries during inhibition of NO synthesis [16]. It is worth noting that we have previously observed that when the cause of disease was still present (L-NAME administration), treatment with the same endothelin antagonist had only a partial alleviating effect on renal morphology despite a quasi-complete inhibition of collagen I gene activation [2]. It appears thus, that correction of the initiating cause (L-NAME removal) contributes more to regression compared to pharmacological treatment against the ETA/B receptors.

It is interesting to note that when endothelin antagonism was combined with L-NAME removal the inhibitory effect on collagen I gene activation was more pronounced in renal preglomerular vessels than glomeruli (Figure 2). It is possible that the endothelin action is predominant in vessels. Supporting this hypothesis, we have observed that immunoreactive endothelin was mainly localized in renal resistance vessels during the development of fibrosis in the L-NAME model [6]. In addition, a similar pattern of response was observed with the aortic tissue: collagen I gene activation was partially blunted after withdrawal of L-NAME, whereas it was completely inhibited with the addition of endothelin antagonist (data not shown).

It is also possible that the addition of endothelin antagonist accelerated the rate of recovery in the tissues in which its action was the most important (vessels), and if the observation period of the recovery had been longer the two groups would completely converge.

Blockers of angiotensin II preserved kidney function and morphology in addition to normalizing systolic pressure in the L-NAME model of hypertension in rats [17,18]. More recently, we have observed that angiotensin II antagonism can induce a regression of
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renal fibrosis and we have proposed that the recovery of renal function resulted from a decrease of de novo synthesis of extracellular matrix (due to the blockade of angiotensin II action) associated to increased matrix degradation (due to metalloproteinase activity) [19]. There are significant differences between the current and our previous studies: one concerns the kinetics (much longer period of L-NAME treatment in mice than in rats) and is apparently a species-due difference. The most important difference concerns the therapeutic strategy: in all previous studies we used pharmacological antagonists of vasoconstrictors (AT1 and/or ETA/B antagonists) which did not correct the cause of the endothelial dysfunction (inhibition of NO synthesis). In contrast, in the present study there was a correction of the initiating cause (inhibition of endogenous NO) that was achieved by L-NAME removal. To integrate the previous to current findings, we propose that the L-NAME model involves rupture of a NO/vasoconstrictor balance (or abnormal activation of vasoconstrictor systems in the absence of endogenous NO). In either case, pharmacological antagonism of vasoconstrictors and/or reactivation of endogenous NO synthesis suppressed the abnormal activation of collagen I and led to regression.

Despite the impressive regression that we have observed with our experimental approach, the issue remains whether there is a no-return point of the progressive decline of renal structure and function. Future studies addressing the issue of defining this point and characterizing its features (urinary or plasma markers, cell phenotype changes) will add valuable information of how to treat regression of renal fibrosis in the different stages of progression. Hope that renal fibrosis is a reversible process in humans came from a study showing regression of renal lesions and improvement of renal function in diabetic nephropathy, 10 years after correction of the initiating cause (normalization of glycaemia after pancreas transplantation) [20].

In conclusion, the present study is among the first reporting a substantial recuperation of renal morphology after a long-term induction of the hypertensive pathology. Improvement of the endothelial dysfunction, the originating cause of the pathology in this model, almost completely corrected the renal structural alterations, providing thus an important advancement for the development of therapeutic strategies against renal chronic failure.

Acknowledgements. This work was financially supported by the ‘Institut National de la Santé et de la Recherche Médicale’ and the ‘Faculté de Médecine St Antoine’; an important part was performed in the technical platform of ‘Explorations fonctionnelles renales de la souris’ of IFR65-St Antoine. Dr J.J. Boffa was research fellow of ‘Académie Nationale de Médecine’.

Conflict of interest statement. None declared.

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Received for publication: 27.5.05
Accepted in revised form: 28.11.05