Vascular Endothelin-1 Gene Expression and Synthesis and Effect on Renal Type I Collagen Synthesis and Nephroangiosclerosis During Nitric Oxide Synthase Inhibition in Rats

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Background—The progression of hypertension during NO deficiency is associated with renal vascular fibrosis due to increased extracellular matrix (mainly collagen I) formation. The purpose of the present study was to investigate whether endothelin-1 (ET-1) is involved in this pathophysiological process.

Methods and Results—Treatment of rats for 4 weeks with the NO synthase inhibitor \(N^\omega\)-nitro-L-arginine methyl ester (L-NAME) 50 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\) increased systolic blood pressure to 159±12 mm Hg. In animals treated with L-NAME, histological evaluation of renal sections revealed an increased formation of extracellular matrix (Masson’s trichrome), and specifically of collagens (Sirius red). A part of this fibrosis was attributed to abnormal collagen I presence, because mRNA expression of the collagen I \(\alpha_1\) chain (reverse transcription–polymerase chain reaction) and procollagen I formation (radioimmunoassay) were increased 3- and 2.5-fold, respectively, in the renal resistance vessels of hypertensive animals. In subsequent experiments, we examined whether ET-1 was involved in activation of collagen I formation. mRNA expression (RNase protection assay) of preproET-1 and ET-1 content (radioimmunoassay) were 10-fold and 3-fold increased, respectively, in renal microvessels of rats treated with L-NAME. Interestingly, in these vessels, ET-1 (immunostaining) was colocalized with sudanophilic lesions. Bosentan, an ET receptor antagonist (20 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\)), coadministered with L-NAME canceled the increased mRNA expression and synthesis of collagen I and attenuated the severity of renal vascular lesions without affecting L-NAME–induced high blood pressure.

Conclusions—These data demonstrate that ET-1 synthesis is increased in renal microvessels when NO production is suppressed. In this model of hypertension, ET-1 is a major activator of collagen I formation in renal resistance vessels and participates in the development of renal fibrosis without affecting systolic blood pressure. (Circulation. 1999;99:2185-2191.)

Key Words: hypertension ■ nitric oxide ■ endothelin ■ kidney ■ fibrosis

Nephroangiosclerosis is a major cause of end-stage renal disease. It is characterized by perivascular collagen I deposition and hypertrophy of smooth muscle cells leading to increased vascular resistance and, finally, glomerular ischemia. In addition to changes in hemodynamics, a strong body of evidence supports the role of endothelial dysfunction, with a particular emphasis on NO pathway impairment, in this process. Several recent studies suggest that activation of endothelin (ET) is associated with a deficiency of the NO pathway. The pathophysiological relevance of such an NO/ET imbalance in the renal angiosclerotic process has still to be demonstrated. ET-1 has mitogenic properties and the ability to regulate extracellular matrix synthesis by vascular smooth muscle cells (VSMCs) in vitro. Because collagen I is almost absent in renal vessels and glomeruli, it is considered to be a marker of pathophysiological process in abnormal vascular remodeling. Therefore, we hypothesized that long-term blockade of vascular NO production would be associated with enhanced renal vascular production of both ET-1 and collagen I. To this end, we measured ET-1 and collagen I mRNA expression and synthesis. We subsequently investigated the role of ET-1 in the in vivo synthesis of collagen I in the renal vessels by studying the effect of the pharmacological blockade of ET-1 receptors during NO deficiency.

Methods

Experimental Protocol
Male Sprague-Dawley rats weighing 120 to 130 g at the beginning of the experiments were maintained on a normal-salt diet with free...
access to chow and tap water. NO synthesis was inhibited with $N^\circ$-nitro-L-arginine methyl ester (L-NAME), an NO synthase inhibitor (50 mg·kg$^{-1}·d^{-1}$). In separate groups of control or L-NAME–treated rats, the nonselective endothelin receptor antagonist bosentan was administered orally (20 mg·kg$^{-1}·d^{-1}$). L-NAME administration gradually elevated systolic blood pressure, which reached a plateau at 4 weeks, in agreement with the literature. Therefore, all subsequent experiments were conducted after 4 weeks of treatment. Control rats were time- and age-controlled with the experimental groups. The protocol followed the European Union guidelines for animal care and protection.

**Measurement of Blood Pressure**

Systolic blood pressure was measured by the tail-cuff method as previously described.

**Isolation of Preglomerular Vessels**

The technique to isolate pregglomerular arterioles was the same as previously described. Vascular preparations containing >90% of pregglomerular vessels were retained for subsequent experiments. The protein vascular content was measured according to Bradford’s method.

**Measurement of Preproendothelin-1 mRNA Expression**

The ribonuclease protection assay was adapted from previous studies. Total RNA was extracted from the vessels with the Trizol kit (Life Technologies–Gibco). For each assay, 40 μg of total RNA was hybridized with the preproET-1 and the GAPDH specific riboprobes. The protected 228- and 164-nucleotide mRNA fragments for preproET-1 or GAPDH were quantified with a phosphorimager–based imaging system, Bas 1000 (Fuji). Results are expressed as the ratio of the β-radioactivity of the preproET-1 to the GAPDH corresponding bands.

**ET-1 Vascular and Urinary Contents**

ET-1 was extracted from pregglomerular vessels according to a method adapted from Matsumoto et al. Pregglomerular arterioles were isolated, resuspended in 1 mol/L acetic acid containing protease inhibitors (Minicomplete, Boehringer), and boiled for 5 minutes. The homogenates were centrifuged for 30 minutes at 12 000g at −4°C. The supernatants were retained for ET-1 radioimmunoassay (RIA) and ultrafiltered (Centriprep-50 columns, Amicon), and 200 μL of the retentate was taken for analysis in a PICP RIA kit (Orion Diagnostica). We used the sequential saturation procedure as described by Oikarinen et al to increase the sensitivity of the assay. Diagnostica). We used the sequential saturation procedure as described by Oikarinen et al to increase the sensitivity of the assay. We calculated and compared with GenBank and the EMBL genomic data bank with the basic local alignment search tool (BLAST) algorithm to verify their identity with the theoretical targets.

**Evaluation of Collagen I Synthesis**

Procollagen I carboxyterminal (PICP) is freed during the extracellular processing of type I procollagen before the collagen molecules form collagen fibers. A stoichiometric ratio of 1:1 exists between the number of collagen molecules produced and that of PICP released.

Freshly isolated pregglomerular arterioles (400 to 900 μg of protein) were incubated at 37°C in a final volume of 0.4 mL of RPMI medium. After 4 hours, aliquots were quickly frozen at −80°C for 30 minutes then melted at room temperature. Supernatants were collected and ultrafiltered (Centriprep-50 columns, Amicon), and 200 μL of the retentate was taken for analysis in a PICP RIA kit (Orion Diagnostica). We used the sequential saturation procedure as described by Oikarinen et al to increase the sensitivity of the assay. We also measured urinary PICP concentration using the above-described kit. PICP values were normalized to urinary creatinine concentration.

**Renal Histology**

Renal tissues from ≥3 rats from each group were fixed in Dubosq solution. Two cortical slices of each kidney were embedded in paraffin after conventional processing (ethanol dehydration and xylene clearing), and 3-μm-thick sections were stained with Masson’s trichrome (staining of extracellular matrix proteins) and Sirius red (staining of collagens).

**Morphological Evaluation**

Sections of kidneys were examined on a blind basis for the degree of glomerular sclerosis and microvascular injury according to the 0 to IV+ injury scale, adapted from established methodology. An injury scale of 0 means no damaged glomeruli, and I, II, III, and IV correspond to 1% to 25%, 26% to 50%, 51% to 89%, and 90% to 100% injured glomeruli, respectively. Thirty to 40 sections (contain-
ing ≥20 glomeruli per sample) were studied in each group of animals.

Statistical Methods
Statistical analysis was performed by ANOVA followed by Bonferroni test of the SigmaStat 2.0 software package. Results with P < 0.05 were considered statistically significant. All values are mean ± SEM.

Results

ET-1 Vascular Production and Urinary Excretion
Figure 1 illustrates preproET-1 mRNA expression in preglomerular vessels in rats treated or not treated with L-NAME. PreproET-1 mRNA expression was almost undetectable in preglomerular vessels of control animals, whereas it was markedly enhanced in vessels from L-NAME–treated rats (2.10 ± 0.85 versus 22.30 ± 8.9 preproET-1/GAPDH cpm ratio, n = 8, P < 0.01). After 4 weeks of L-NAME treatment, ET-1 content was 2- to 2.5-fold increased in preglomerular arterioles (12.26 ± 0.43 versus 5.10 ± 0.26 pg/mg, n = 6, P < 0.01; Figure 2) and in urine (2.96 ± 0.16 versus 1.49 ± 0.3 pg/μmol creatinine, n = 6, P < 0.01; Figure 3). ET-1 immuno-

staining, negative in controls, revealed a weak (4 weeks of L-NAME treatment), then a more marked staining in renal resistance vessels (6 weeks, Figure 4). Interestingly, detection of irET-1 was colocalized mainly with sudanophilic lesions at the junction of interlobular and afferent arterioles.

Systolic Blood Pressure
Systolic blood pressure was significantly higher in rats after 4 weeks of L-NAME treatment than in controls (159 ± 12 versus 116 ± 8 mm Hg, P < 0.01, n = 15) (Figure 5). Bosentan had no effect on baseline systolic blood pressure (116 ± 8 versus 119 ± 11 mm Hg in controls versus bosentan-treated rats, respectively, n = 15). Similarly, bosentan did not change the increased systolic blood pressure of L-NAME–treated rats at 4 weeks (159 ± 12 versus 158 ± 7 mm Hg in rats with and without bosentan, respectively, n = 15).

Collagen I Production
Because collagen I is a major component in the development of vascular fibrosis,8 we examined the ability of renal vessels to produce collagen I during chronic NO synthase inhibition. Synthesis of PICP was higher in renal resistance vessels of L-NAME–treated rats than in control rats (4.24 ± 0.27 versus 2.38 ± 0.17 ng/mg, n = 7, P < 0.05; Figure 6). The increase in collagen I synthesis was a pretranslational event, because NO-deficient hypertensive rats exhibited more vascular

Figure 3. ET-1 excretion rate in urine in rats treated with L-NAME during 4 weeks. Values are mean ± SEM of 2 experiments, n = 6 per group. *P < 0.05 vs control.

Figure 4. Representative example of ET-1 immunostaining in interlobular (IL) and afferent arterioles (AA) of rats treated with L-NAME. Controls were negative (not shown). Note increased staining in junction of IL and AA and focal increased staining in IL and AA (arrows). Bar = 50 μm.

Figure 2. ET-1 content (measured by RIA) in preglomerular vessels of L-NAME–treated rats. Values are mean ± SEM of 3 experiments (6 rats per group). *P < 0.05 vs control.

Figure 1. Top, Representative display of a ribonuclease protection assay experiment (phosphoimager recording). Bottom, PreproET-1 mRNA expression in preglomerular vessels in rats treated with L-NAME for 4 weeks. Values are mean ± SEM of preproET-1 vs GAPDH mRNA signal ratio. Two independent experiments, n = 8 rats per group. *P < 0.05 vs control.
mRNA expression of the procol1(I) gene than control animals [2.28±0.02 versus 0.81±0.01 procol1(I)/GAPDH signal ratio, n=7, P<0.05; Figure 7]. In control animals, bosentan administration did not affect procol1(I) mRNA expression [0.77±0.02 versus 0.81±0.01 procol1(I)/GAPDH signal ratio, n=6 per group, Figure 7] and PICP synthesis (2.25±0.10 versus 2.38±0.17 ng/mg, n=6 per group, Figure 6) in renal resistance vessels. In contrast, bosentan prevented the activation of procol1(I) gene induced after 4 weeks of L-NAME [2.28±0.02 versus 1.07±0.04 procol1(I)/GAPDH signal ratio, n=9, P<0.05, for L-NAME– and L-NAME+bosentan–treated animals, respectively, Figure 7]. Similarly, bosentan completely canceled the increase in PICP synthesis induced by L-NAME (4.24±0.27 versus 2.59±0.28 ng/mg, n=6, P<0.05 for L-NAME– and L-NAME+bosentan–treated animals, respectively, Figure 6).

Bosentan administration also prevented the 2-fold increase in PICP urinary excretion rate in L-NAME–treated rats (3.76±0.28 versus 6.8±0.65 and 3.80±0.51 ng/µmol creatinine for controls, L-NAME alone–, and L-NAME+bosentan–treated animals, respectively, n=5, P<0.05).

Renal Fibrosis and Collagen Deposition

Increased levels of extracellular matrix formation were detected after 4 weeks of L-NAME treatment in glomeruli and small vessels stained with the Masson’s trichrome method, indicating induction of renal vascular and glomerular fibrosis (Figure 8A through 8C). In these vessels, at least a part of extracellular matrix deposition was due to collagen synthesis, as revealed by staining with Sirius red, an agent that specifically labels collagens (Figure 8D through 8F). ET-1 antagonism markedly protected the kidneys from the L-NAME–induced fibrosis, as evidenced by the small amounts of extracellular matrix revealed by Masson’s trichrome and the low levels of collagen staining by Sirius red. As shown in the Table, 10.3% of renal sections of L-NAME+bosentan–treated animals showed mild injury (grade II), whereas 14.4% and 81.2% of sections (grade III and IV, respectively) showed severe damage in the L-NAME–treated group. The histological injury score was markedly lowered by endothelin antagonism in hypertensive animals (P<0.001).

Discussion

The model of hypertension induced by chronic NO inhibition is characterized by the severity of the renal lesions. Recent studies indicate that at least part of this pathological process is independent of changes in hemodynamics and could be attributed to the role of locally formed profibrogenic factors. The purpose of the present study was to examine whether ET-1 is involved in the development of renal vascular fibrosis observed during NO deficiency. Several in vitro and in vivo studies suggest that ET-1 synthesis is activated during impairment of the NO pathway. PreproET-1 gene expression rate was repressed by NO donors and increased by L-NAME in cultured human umbilical vascular endothelial cells. NO inhibited the synthesis and/or the vasoconstrictor effects of ET-1 in vivo. Tuning of NO/ET-1 balance also regulates the VSMC phenotype in vivo. In contrast, other investigators did not observe increased levels of ET-1 mRNA or peptide in aortas of rats

Figure 5. Systolic blood pressure measured in adult Sprague-Dawley rats treated with L-NAME (50 mg · kg⁻¹ · d⁻¹) for up to 4 weeks. Values are mean±SEM of 15 rats per group. *P<0.05 vs control.

Figure 6. Effect of long-term treatment with bosentan, an ET receptor antagonist (20 mg · kg⁻¹ · d⁻¹), on PICP vascular production in rats treated or not treated with L-NAME during 4 weeks. Values are mean±SEM of 2 experiments, n=6 or 7 per group. *P<0.05 vs control. #P<0.05 vs L-NAME alone.

Figure 7. Top, Representative display of an RT-PCR experiment from 1 µg of total RNA extracted from preglomerular vessels of control rats (A), rats treated with L-NAME (B), and rats treated with L-NAME+bosentan (C). Bosentan alone was not different from control (not shown). Bottom, Effect of long-term treatment with bosentan (20 mg · kg⁻¹ · d⁻¹) on procol1(I) mRNA expression by RT-PCR in preglomerular vessels in rats treated with L-NAME. Results are expressed as ratio of optical density of procol1(I) RT-PCR product on coamplified GAPDH product. Values are mean±SEM of 3 experiments (n=9 per group). *P<0.05 vs control. #P<0.05 vs L-NAME alone.
chronically treated with L-NAME. However, as pointed out in these previous reports, aortic ET-1 synthesis may not reflect alterations of ET-1 levels in renal resistance vessels. Our study clearly indicates that ET-1 synthesis and release is activated in renal resistance vessels of rats treated chronically with L-NAME. Renal vascular expression of preproET-1 mRNA was considerably augmented in hypertensive rats compared with controls. In addition, the ET-1 peptide content was increased in preglomerular vessels of L-NAME–treated rats. This activation of ET-1 production in renal arterioles was accompanied by an increased urinary ET-1 excretion rate. Although less sensitive than these methods, ET-1 immunostaining confirmed these biochemical measurements. Marked immunostaining of preglomerular vessels in rats treated with L-NAME during 6 weeks contrasted with the negativity of controls and the weak positivity after 4 weeks. This result suggests that ET-1 renal vascular production goes on increasing during NO deficiency. Immunoreactive ET-1 seems to be localized in VSMCs, which can be the consequence of ET-1 production by these cells or ET-1 binding to its specific receptors. Our data do not allow us to discriminate whether the site of activated ET-1 synthesis is endothelial or by VSMCs. Usually, ET-1 is synthesized by endothelium. However, activated VSMCs could also produce ET-1, as suggested by results from cultured cells in vitro and from atherosclerotic plaques in vivo.

**Semiquantitative Data of Histological Injury Score of Controls and Chronically NOS-Blocked Rats With or Without Endothelin Antagonist Treatment**

<table>
<thead>
<tr>
<th>Injury Scale</th>
<th>Control</th>
<th>L-NAME*</th>
<th>Bosentan</th>
<th>L-NAME + Bosentan†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>94.9±3.6%</td>
<td>...*</td>
<td>95.1±4.0%</td>
<td>19.4±0.1%*†</td>
</tr>
<tr>
<td>I</td>
<td>5.1±0.1%</td>
<td>...*</td>
<td>4.9±0.1%</td>
<td>70.3±7.3%*†</td>
</tr>
<tr>
<td>II</td>
<td>...</td>
<td>4.5±13.3%*</td>
<td>...</td>
<td>10.3±7.2%*†</td>
</tr>
<tr>
<td>III</td>
<td>...</td>
<td>14.4±6.4%*</td>
<td>...</td>
<td>...*†</td>
</tr>
<tr>
<td>IV</td>
<td>...</td>
<td>81.1±6.0%*</td>
<td>...</td>
<td>...*†</td>
</tr>
</tbody>
</table>

Injury scale: 0=no injury; I=up to 25% of the glomeruli involved; II=up to 50% involvement; III=up to 75% involvement; IV=up to 100% involvement. Values are expressed as mean±SEM. *P<0.05 treated rats vs controls. †P<0.05 L-NAME + bosentan vs L-NAME alone.
Several studies revealed a major role of ET-1 in the mechanisms of hypertension in various experimental models depending on angiotensin II activation or the lack thereof. However, it appears that this is not the case in the NO synthase blockade model, because antagonists of ET-A and/or ET-B receptors have no effect on the rise of blood pressure during chronic treatment with L-NAME. Our results are in agreement with these observations, because bosentan, a mixed antagonist of endothelin receptors, did not prevent the L-NAME–induced increase of blood pressure. Because ET-1 synthesis was activated in renal resistance vessels, it might be hypothesized that the mechanisms modulating the renal vascular phenotype were not necessarily the same as those that increased blood pressure. Thus, we thought it of interest to examine whether the increase of ET-1 synthesis affecting the renal resistance vessels explained the marked renal vascular disease observed in L-NAME–treated rats. In the present study, renal fibrosis of L-NAME–treated rats was demonstrated by histological examination. Conventional morphology showed an increase of extracellular matrix in glomeruli and renal cortical interstitium. Staining with Sirius red indicated the abnormal presence of collagen in microvessels. Biochemical parameters allowed us to identify the presence of collagen I. Compared with controls, expression of collagen I mRNA was significantly augmented in preglomerular arteries of L-NAME–treated rats. This overexpression of collagen I is more likely from VSMCs rather than from fibroblasts, because in renal preglomerular arterioles, fibroblasts are very few. However, an involvement of interstitial fibroblasts in the synthesis of perivascular extracellular matrix during the pathophysiological process cannot be excluded. PICP is considered to be a marker of cardiovascular collagen I–mediated fibrosis. In our study, PICP release by renal arterioles was increased in hypertensive rats, which implies that at least a part of the increased extracellular matrix and collagen formation observed by histology was due specifically to collagen I deposition. This activation of collagen I expression was also reflected in the increased urinary excretion rate of PICP in L-NAME–treated animals. Thus, PICP urinary excretion rate seems to be a good indicator of severe damage of renal structure.

In rats treated with bosentan alone, the biochemical and morphological parameters of the extracellular matrix and, more specifically, of collagen I were unchanged compared with controls. This observation suggests that in the presence of NO, ET-1 does not play a substantial role in renal matrix synthesis, most likely because NO exerts a tonic inhibitory control on ET-1 synthesis in the renal vasculature. This mechanism could be reinforced by an NO-mediated down-regulation of ET-1 receptors or an inhibitory effect of NO on the transduction of the signal of vasoconstrictive peptides in their target cells. In hypertensive rats treated with L-NAME, in which renal arteriolar ET-1 synthesis was activated, coadministration of bosentan almost completely prevented the increase of collagen I in preglomerular vessels and markedly limited renal cortical damage. These data corroborate and complete our initial results, in which bosentan completely canceled the L-NAME–induced activation of the procoI promoter. Moreover, the colocalization of ET-1 immunostaining with sudanophilic lesions along the renal resistance vessels suggests that an autocrine or paracrine loop mediates the sclerotic process. Such vascular lesions were previously related to ET action because they were no longer present in bosentan-treated animals.

Our results emphasize the predominant influence of ET-1 activity on the sclerotic process observed in the kidneys of L-NAME–treated rats. ET-1 plays a minor or a significant role in the local response to injury, depending on the vascular beds and the mechanism of injury. For instance, in rats treated with L-NAME, bosentan only slightly modified the medial-lumen ratio of basilar and mesenteric arteries. In contrast, in experimental hepatic fibrosis, the same drug reduced collagen I mRNA expression. Similarly, transgenic mice overexpressing the human ET-1 gene developed glomerulosclerosis and renal interstitial fibrosis. In addition, antagonism of endothelin receptors delayed the progression of renal failure, as evidenced by measurement of urinary protein and histological analysis, and increased the survival rate of rats with renal mass reduction, a model characterized by an impairment of renal NO production.

In conclusion, our study establishes that, in preglomerular vessels, endogenous NO not only acts as a vasodilatory autacoid but also prevents the pathogenic activity of ET-1. This effect of NO is at least partially due to the inhibition of vascular ET-1 synthesis. The interaction between the two autacoids is independent of systemic blood pressure. In the absence of NO, ET-1 plays a major role in the formation of collagen I in the renal resistance vasculature and, more generally, in the development of nephroangiosclerosis. Another salient conclusion of this study is that equivalent levels of systolic blood pressure can be associated with very different renal structural damages, according to the activity of local vasomotor agents. This observation highlights the difficulties in preventing the progression of nephroangiosclerosis and glomerulosclerosis in human hypertension with conventional therapies, even if ACE inhibitors seem partially active. New drugs, particularly ET-1 antagonists, could protect the kidneys in human essential hypertension independently of their antihypertensive efficiency, especially in case of impaired endothelial NO synthesis. Further experimental and clinical studies are needed before we can draw conclusions on the curative effects of ET-1 antagonists on the renal structural damage induced by hypertension.

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