Chronic inhibition of NOS-2 ameliorates renal injury, as well as COX-2 and TGF-β1 overexpression in 5/6 nephrectomized rats

Pablo Bautista-García1, Laura Gabriela Sánchez-Lozada1, Magdalena Cristóbal-García1, Edilia Tapia1, Virgilia Soto2, Ma. Carmen Ávila-Casado2, Ricardo Márquez-Velasco3, Rafael Bojalil3, Martha Franco1 and Jaime Herrera-Acosta1,*

1Department of Nephrology, 2Department of Pathology and 3Department of Immunology, Instituto Nacional de Cardiología Ignacio Chávez, Mexico City, Mexico

Abstract

Background. Chronic renal damage is associated with inflammatory infiltration, fibrosis and vascular lesion, coupled with increased expression of cyclo-oxygenase 2 (COX-2) and transforming growth factor-β1 (TGF-β1). However, the role of inducible nitric oxide synthase (NOS-2) is still controversial. Thus, we studied the contribution of NOS-2 to the expression levels of COX-2 and TGF-β1, as well as the structural renal injury in rats with subtotal renal ablation (5/6 Nx).

Methods. Four groups of rats were studied: sham, 5/6 Nx, 5/6 Nx + aminoguanidine (AG) and 5/6 Nx + L-NIL (L-N6-iminoethyl-lysine). Systolic blood pressure (SBP), proteinuria and creatinine (Cr) clearance were measured. NOS-2, COX-2 and TGF-β1 gene expression was determined by real-time reverse transcription–polymerase–chain reaction. Protein expression was evaluated by western blot and ELISA (TGF-β1). Immunohistochemistry and morphometry were performed for NOS-2, microvascular thickening and fibrosis.

Results. Systemic hypertension and marked proteinuria, increased expression of NOS-2, COX-2 and TGF-β1, thickening of arteriolar wall and tubulointerstitial fibrosis were produced in 5/6 Nx rats. Chronic inhibition of NOS-2 did not prevent arterial hypertension or the fall in Cr clearance, but partially reduced proteinuria. Nevertheless, AG and L-NIL preserved arteriolar morphology and the administration of both selective inhibitors of inducible NOS (AG and L-NIL) prevented NOS-2 overexpression.

Conclusion. This study shows that NOS-2 was markedly enhanced in renal tissue of 5/6 Nx rats. Moreover, treatment with AG and L-NIL prevented the morpho-functional changes induced by subtotal renal ablation, despite persistence of systemic hypertension, suggesting that high concentrations of nitric oxide produced by NOS-2 could act as a positive modulator of the proinflammatory and profibrotic pathways involved in the progression of renal disease.

Keywords: aminoguanidine and L-NIL; COX-2; 5/6 nephrectomy; NOS-2; TGF-β1

Introduction

In the course of chronic renal diseases, the initial insult leads to progressive decline in glomerular filtration rate and eventual loss of the kidney function. Brenner et al. [1] postulated that compensatory adaptations induced by nephron loss produce glomerular hypertension, which results in glomerular lesion, proteinuria, inflammatory infiltration, and, finally, renal scarring. The inflammatory process that takes place between the initial insult and the final process of renal scarring seems to play an important role in the onset of renal disease. In fact, different approaches used to ameliorate inflammation in rats with subtotal renal ablation (5/6 Nx) reduced proteinuria and structural renal injury without lowering systemic hypertension [2,3]. In addition, we demonstrated that suppression of inflammation in 5/6 Nx rats with mycophenolate mofetil prevented arteriopathy despite persistence of systemic hypertension, suggesting that vascular injury contributes to the progression of renal disease [4].

On the other hand, some studies have shown that renal damage is associated with increased expression of inducible nitric oxide synthase (NOS-2) in glomeruli and renal interstitium, which could enhance the generation of oxidative damage by the reactive nitrogen intermediates [5,6]. However, there is controversy about the participation of NOS-2 in renal...
found a significant decrement of NOS-2 renal expression in rats with subtotal renal ablation. On the other hand, Fujihara et al. [8], using antibodies targeted against two different epitopes, found the presence of what appeared to be two distinct NOS-2 fractions: a ‘tubular’ fraction, present in sham rats and less intensely in 5/6 Nx; and an ‘interstitial’ fraction, observed only in swollen areas of the 5/6 Nx group. Although the cell type that expressed this fraction was not reported, the authors suggested that it was probably synthesized by interstitial inflammatory cells. In fact, treatment with the NOS-2 specific inhibitor, aminoguanidine (AG) greatly attenuated renal injury in 5/6 Nx rats by a direct anti-inflammatory effect, probably related to NOS-2 inhibition [9,10].

Large quantities of nitric oxide (NO) produced by NOS-2 have been related to the generation of peroxynitrite, activation of proinflammatory proteins such as cyclo-oxygenase (COX-2) through interaction with the heme moiety of the protein [11], and activation of profibrotic molecules such as transforming growth factor-β (TGF-β), by the suppression of TGF-β latency associated-peptide (LAP) activity [12]. Thus, through these effects, NO may participate in the accumulation of the extracellular matrix during chronic renal damage.

Thus, functional, vascular and structural renal damage may be partially mediated through increased expression of COX-2 and TGF-β1, stimulated by NO produced by NOS-2. In the present study, we evaluated in 5/6 Nx rats if the renoprotective effect exerted by chronic inhibition of NOS-2 with two different selective inhibitors of NOS-2 (L-NIL and AG) is associated with prevention of the expression and/or activation of COX-2 and TGF-β1.

Methods

Adult male Sprague–Dawley rats (300–350 g) were obtained from Harlan, Mexico. Thirty rats underwent 5/6 Nx by the removal of the right kidney and selective infarction of approximately two-thirds of the left kidney by ligation of two or three branches of the renal artery [4]. Fifteen sham-operated rats served as control. Experimental procedures were conducted according to our institutional guidelines.

Experimental groups

Rats were randomly assigned to four groups: S (n = 15), sham-operated rats, 5/6 nephrectomy (Nx, n = 15), Nx rats plus AG (n = 15, AG, Sigma–Aldrich, Mexico, 0.1% in drinking water). To determine whether the beneficial effects of AG on NOS-2, COX-2 and TGF-β1 mRNA levels were actually due to selective inhibition of NOS-2 or due to side effects of the compounds, we performed real-time reverse transcription–polymerase chain reaction (RT–PCR) in an additional group of rats in which the specific inhibitor of NOS-2 (L-N6-iminoethyl-lysine: L-NIL 30 mg/l in drinking water) was given. All groups of rat were studied after 21 days of follow-up.

Systolic blood pressure and proteinuria

Both parameters were measured in the basal period and at the end of the study. Systolic blood pressure (SBP), in conscious restrained rats, was measured by tail cuff sphygmomanometer using an automated system (Narco Biosystems, Houston, TX, USA). All animals were preconditioned for blood pressure measurements 1 week before each experiment. Twenty-four hour urine was collected for determination of proteinuria by a turbidimetric method [4]. In five rats of each group, serum and urine Cr concentration were measured with an autoanalyzer (Creatinine Analyzer 2, Beckman Instruments, Inc., Fullerton, CA, USA), and renal Cr clearance was calculated by the standard formula $C = UV/P$, where $U$ is the concentration in urine, $V$ is urine flow rate, and $P$ is the plasma concentration.

Relative quantification of NOS-2, COX-2 and TGF-β1 genes

Total mRNA was isolated from individual cortexes and medullas (n = 5 rats each group) as previously described [13].

The purified RNA (5 μg) was reverse-transcribed using oligo-dT (0.5 μg) and 200 U of M-MLV Reverse Transcriptase (Invitrogen Life Technologies) for 1 h at 37°C.

Relative mRNA levels of NOS-2, COX-2 and TGF-β1 were assessed in sham, 5/6 Nx, 5/6 Nx + AG and 5/6 Nx + L-NIL groups by real-time RT–PCR with a LightCycler 2.0 System (Roche Applied System) using Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen Life Technologies) which was used according to the manufacturer’s protocol. Samples were then amplified for NOS-2, COX-2, TGF-β1 and β-actin under the following conditions: Pre-incubation for 10 min at 95°C, denaturation for 10 s at 95°C, annealing for 10 s at 60°C and extension step for 12 s at 72°C, 40 cycles. Sequences of primers were as follows (Invitrogen Life Technologies): NOS-2 [14]: 5’ TGC ATG GAC CAGTAT AAG GCA AAC 3’ (forward), 5’ GTT TCT GGT CGT CAT GAG CAA (reverse), for COX-2 [15]: 5’ GAA ATG GCT GCA GAG TTG (forward), 5’ TCA TCT AGT CTG GAG TGG 5’ (reverse) for TGF-β1 [16]: 5’ CAC CAT CCA TGA CAT GAA CC 3’ (forward), 5’ TCA TGT TGG ACA ACT GCT CC 3’ (reverse), and β-actin, as reported previously [17].

The results were quantified as Ct values, where Ct is defined as the threshold cycle of PCR at which the amplified product is first detected, and expressed as relative gene expression (the ratio of target/β-actin) [18].

Western blotting

Western blot analysis was performed with whole kidney tissue homogenates. For analysis of COX-2, microsomes were isolated as previously described [19], and for NOS-2 analysis, a supernatant was used (n = 5 each group). Blots were probed with NOS-2 (1:200 dilution) (BD Transduction Laboratories), COX-2 (1:100 dilution), and β-actin (1:300 dilution) antibodies (Santa Cruz Biotechnology, Inc.), the secondary antibodies (1:7000) (ECL Plus Western Blotting
Renal ablation induced a significant fall of Cr clearance in treated and untreated 5/6 Nx rats (Sham: 1.33 ± 0.17 ml/min; 5/6 Nx: 0.57 ± 0.10 ml/min; 5/6 Nx + AG: 0.64 ± 0.06 ml/min. P < 0.01 vs sham).

Expression of NOS-2
As shown in Figure 1, 5/6 Nx induced a significant overexpression of NOS-2 mRNA in renal cortex and medulla (1.3- and 3.3-fold, respectively compared with 5/6 Nx rats), which was prevented by both AG and L-NIL treatments. Likewise, expression of NOS-2 protein in total renal tissue evaluated by western blot was significantly increased in 5/6Nx rat; which was prevented by AG treatment (Figure 1C–D). Using the same antibody, immunohistochemistry revealed that overexpression of the NOS-2 protein was mainly located in the tubulointerstitial areas, and by morphometry we found a significant increase in 5/6 Nx groups (Sham: 1357 ± 48; 5/6 Nx: 6370 ± 407 pixels/400 x field, P < 0.001 vs sham, Figure 1E–F). Up-regulation of NOS-2 was prevented in the 5/6 Nx + AG group as well as in L-NIL treated rats (3296 ± 18 and 2818 ± 64 pixels/400 x field respectively, P < 0.001 vs 5/6 Nx) (Figure 1G–H).

Expression of COX-2 and TGF-β1
Subtotal renal ablation also induced an increase of COX-2 mRNA in renal cortex and medulla (1.6- and 2.0-fold, respectively compared with 5/6 Nx group), which was prevented by both AG and L-NIL treatments (Figure 2A–B). In addition, immunoblot showed a significant increase of COX-2 protein in the 5/6 Nx group, which was prevented by the AG treatment (Figure 2C–D). Similarly, 5/6 Nx increased TGF-β1 mRNA in cortex and medulla (1.3- and 1.8-fold compared with 5/6 Nx rats); this increment was also prevented by AG and L-NIL treatments (Figure 3A–B).

Table 1. Body weight, systolic blood pressure (SBP) and proteinuria (Uprot) of rats chosen to study gene expression (real-time RT–PCR) and protein expression (western blot, WB; ELISA and immunohistochemistry, IMHC)

<table>
<thead>
<tr>
<th>Method</th>
<th>Parameter</th>
<th>Sham</th>
<th>5/6 Nx</th>
<th>5/6 Nx + AG</th>
</tr>
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<tbody>
<tr>
<td>RT–PCR</td>
<td>Body weight</td>
<td>306 ± 2</td>
<td>304 ± 9</td>
<td>291 ± 11</td>
</tr>
<tr>
<td>n = 5 each group</td>
<td>SBP</td>
<td>124 ± 3</td>
<td>164 ± 9*</td>
<td>164 ± 4**</td>
</tr>
<tr>
<td></td>
<td>Uprot</td>
<td>7 ± 1</td>
<td>114 ± 6*</td>
<td>57 ± 13***</td>
</tr>
<tr>
<td>WB and ELISA</td>
<td>Body weight</td>
<td>306 ± 3</td>
<td>305 ± 12</td>
<td>290 ± 13</td>
</tr>
<tr>
<td>n = 5 each group</td>
<td>SBP</td>
<td>108 ± 3</td>
<td>163 ± 8†</td>
<td>156 ± 6*</td>
</tr>
<tr>
<td></td>
<td>Uprot</td>
<td>6 ± 0.3</td>
<td>73 ± 3‡</td>
<td>47 ± 3†</td>
</tr>
<tr>
<td>IMHC</td>
<td>Body weight</td>
<td>304 ± 4</td>
<td>311 ± 6</td>
<td>294 ± 11</td>
</tr>
<tr>
<td>n = 5 each group</td>
<td>SBP</td>
<td>109 ± 3</td>
<td>175 ± 14†</td>
<td>153 ± 5*</td>
</tr>
<tr>
<td></td>
<td>Uprot</td>
<td>10 ± 2</td>
<td>96 ± 11*</td>
<td>67 ± 5†</td>
</tr>
</tbody>
</table>

*P < 0.01 vs 5/6 Nx; **P < 0.01 vs sham; †P < 0.001 vs sham; ‡P < 0.001 vs 5/6 Nx; ††P < 0.05 vs 5/6 Nx.

Results
Systolic blood pressure and proteinuria
Body weight, SBP and proteinuria of each group of rats chosen to study RT–PCR, western blot and immunohistochemistry are depicted in Table 1. At 21 days of follow-up, groups with renal ablation had a marked elevation of SBP and proteinuria compared with sham rats. Treatment with AG did not prevent the rise in arterial pressure but partially prevented proteinuria. L-NIL treatment in 5/6 Nx rats had a comparable behaviour to AG since it also significantly reducing proteinuria (33 ± 7 mg/day, P < 0.001 vs 5/6 Nx) while SBP remained elevated (153 ± 5 mmHg, P = ns).

Renal histology
In five rats of each group, remnant kidneys were washed by perfusion with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde, embedded in paraffin, and 4 μm sections were stained with periodic acid Schiff (PAS) reagent. Afferent arteriole morphology, evaluated as the media-to-lumen ratio, was assessed by indirect peroxidase immunostaining for α-smooth muscle actin (α-SMA, Dako Corp, Carpinteria, CA, USA) as described elsewhere [20]. Tubulointerstitial fibrosis was detected by Masson staining. NOS-2 expression was evaluated by the indirect streptavidin-biotin-peroxidase method, counterstained with haematoxylin, using the same antibody as in western blot. Ten non-crossed fields of cortex (10X) per biopsy were analysed using Image-Pro-Plus 5.0 (Media Cybernetics, Silver Spring, MD, USA) and Adobe Photoshop 7 (Adobe Systems, San Jose, CA, USA). Positive brown-colour areas were selected and quantified in pixel units and the number of positive areas were expressed as a fraction of the total area (positive brown areas divided by overall field area). The mean fractional amount of positive brown areas was obtained by averaging the values from 10 examined fields.

Measurement of TGF-β1
TGF-β1 was measured in plasma samples of rats used for western blot (n = 5 each group) with a commercially available enzyme-linked immunosorbent assay kit (Quantikine, R&D System, Minneapolis, MN, USA) following the manufacturer’s instructions.

Statistical analysis
Values were expressed as mean ± SE. Differences between groups were evaluated by analysis of variance (ANOVA) with appropriate correction for multiple comparisons (Bonferroni). The relation between variables was assessed by correlation analysis.
Fig. 1. NOS-2 renal expression. Cortical (A) and medullary (B) gene expression evaluated by real-time RT–PCR. Representative western blot in renal homogenates (C, D). NOS-2 immunohistochemistry (400x; E–H). A significant increase of NOS-2 renal expression in 5/6 Nx rats was observed in tubular epithelial cells, which was prevented in AG and L-NIL treated groups. *P < 0.05 vs sham; **P < 0.05 vs 5/6 Nx.

Fig. 2. COX-2 renal expression. Gene expression evaluated by real-time RT–PCR in cortex (A) and medulla (B). Protein expression was evaluated by western blot in renal microsomes (C, D). C is a representative western blot of COX-2. *P < 0.05 vs sham; **P < 0.05 vs 5/6 Nx.
The active fraction of the TGF-β1 protein was evaluated by ELISA. Renal ablation induced a marked increase of the active fraction of TGF-β1 compared with sham rats (16.71 ± 3.67 ng/ml vs 3.51 ± 0.75, \( P < 0.05 \)), which was prevented in the 5/6 Nx + AG group (4.62 ± 0.82 ng/ml, \( P < 0.05 \) vs 5/6 Nx) (Figure 3C).

**Histological studies**

Quantification of tubulointerstitial fibrosis by morphometry showed increased fibrosis in subtotal renal ablated rats compared with the sham group (4653 ± 362 vs 1940 ± 107 pixels/area, \( P < 0.05 \)). This morphological change was prevented by the administration of AG and L-NIL (2751 ± 181 and 2245 ± 98 pixels/area, respectively, \( P < 0.05 \) vs 5/6 Nx) (Figure 4A, C, E and G).

We previously showed that 5/6 Nx induces a renal vascular lesion characterized by arteriolar wall thickening and narrowing of the vascular lumen [6]. In the present study, we found a similar microvascular lesion as disclosed by a significant increment in the media-to-lumen ratio compared with sham rats (M/L = 6.37 ± 0.59 vs 3.55 ± 0.20, \( P < 0.01 \)). The arteriolopathy was prevented by the administration of AG and L-NIL (M/L = 3.47 ± 0.18 and 3.22 ± 0.08, respectively, \( P < 0.01 \) vs 5/6 Nx) (Figure 4B, D, F and H).

**Correlations**

We analysed the results of NOS-2 expression obtained by different approaches and examined its correlations among the studied parameters. These results are shown in Table 2. Cortical NOS-2 gene expression positively correlated with proteinuria, COX-2, and TGF-β1.

![Fig. 3. TGF-β1 expression. Gene expression was evaluated by real-time RT-PCR in renal cortex (A) and medulla (B). Protein active fraction of TGF-β1 was evaluated by ELISA in plasma (C). *\( P < 0.05 \) vs sham; **\( P < 0.05 \) vs 5/6 Nx.](https://academic.oup.com/ndt/article-abstract/21/11/3074/1876624)

![Fig. 4. Micrographs showing tubulointerstitial fibrosis (Masson, 400×) (A, C, E) and microvascular damage (PAS and α-SMA immunostaining, 1000×) (B, D, F) in sham (A, B), 5/6 Nx (C, D) and 5/6 Nx + AG (E, F) and L-NIL (G, H) rats.](https://academic.oup.com/ndt/article-abstract/21/11/3074/1876624)
By western blot, NOS-2 correlated with proteinuria and COX-2, and also with the active fraction of TGF-β1 measured by ELISA. Morphometric analysis in groups used for immunohistochemistry revealed positive correlations among NOS-2 vs proteinuria, media-to-lumen ratio and fibrosis.

### Discussion

Several studies have shown that during chronic renal injury, inhibition of inflammatory infiltration prevents or retards haemodynamic and structural alterations [2–4]. In this context, participation of NOS-2 may be relevant, since this isoform is induced in infiltrating inflammatory cells, and its high and continuous activity may have deleterious effects on renal tissue. However, the contribution of NOS-2 to the progression of renal damage is still controversial. In this regard, Aiello et al. [21] were the first to report decreased expression of NOS-2 in kidney sections of 5/6 Nx rats, and later on Vaziri et al. [7] confirmed this result in whole kidney homogenates. On the other hand, Brooks et al. [9] found in the same experimental model that administration of AG, a generally accepted specific inhibitor of NOS-2 [22], attenuated proteinuria. More recently, Fujihara et al. [8], suggested the existence of two functionally distinct NOS-2 renal fractions in 5/6 Nx rats: a constitutive tubular fraction and an interstitial fraction, associated with interstitial inflammation.

In the present study, we evaluated the participation of NOS-2 during chronic renal damage using three different approaches. In order to inhibit NOS-2, 0.1% of AG or 30 mg/l of L-NIL were added to drinking water. It had been previously shown that 0.1% of AG successfully blocks NOS-2, not inhibiting endothelial NOS or the formation of glycation end products (AGEs) [23]. Although the inhibition of NOS-2 by AG is considered to be specific, it can also reduce advanced AGEs formation, however presence of AGEs in 5/6 Nx rats was reported only after 12 weeks post-Nx [24]. Thus, it is unlikely that inhibition of AGEs production by AG had contributed to the beneficial effects exerted by AG in the present study performed after 3 weeks of subtotal renal ablation. Nevertheless, in order to corroborate if the beneficial effect of AG treatment is actually due to selective inhibition of NOS-2, we used a more selective inhibitor of NOS-2, L-NIL. We found that both NOS-2 inhibitors prevented the overexpression of NOS-2, COX-2 and TGF-β1 mRNA suggesting that both AG and L-NIL exerted their therapeutic effects through the inhibition of inducible NOS. In this regard, it was shown that AG and L-NIL blockade the action of NOS-2 as an analogue substrate, indicating post-translational, rather than transcriptional inhibition [22]. However, the observation that AG and L-NIL also limit the expression of mRNA levels of NOS-2, suggests that both inhibitors may have effects on its transcriptional activation. Although, we can rule out a direct effect of AG and L-NIL on mRNA NOS-2 expression levels, it is likely that the decrement of NO and its oxidative radicals mediated by AG and L-NIL may prevent the activation of potent endogenous inducers such as cytokines, interferon-γ or tumour necrosis factor-α, produced during an inflammatory process, which could enhance the translocation of free NF-κB from the cytosol to the nucleus and the induction of NOS-2 expression [25]. In support of a negative effect of AG and L-NIL on transcriptional activation of NOS-2, we found that protein expression measured as western blot was prevented in AG treated rats compared with 5/6 Nx rats and protein expression levels measured as immunohistochemistry were also decreased in AG and L-NIL treated rats compared with 5/6 Nx group.

Since progression of renal damage is partially mediated by proteinuria, proinflammatory and profibrotic molecules as well as structural damage, we studied if NOS-2 overexpression is related to these alterations, by analysis of expression of COX-2 and TGF-β1, proteinuria, tubulointerstitial fibrosis and arteriolar thickening. Our results suggest that increased NOS-2 expression induced overexpression of COX-2 and TGF-β1 at gene and protein levels. This is supported by the fact that NOS-2 inhibition with AG and L-NIL prevented the increased expression of both mRNAs. In fact, we found a positive correlation between expression of NOS-2 and COX-2 by real-time RT–PCR and western blot, and NOS-2 and TGF-β1 by real-time RT–PCR and protein expression (NOS-2, western blot and TGF-β1, ELISA). It has been postulated that tubulointerstitial fibrosis and vascular damage may result from the enhanced expression of COX-2 [26] and TGF-β1 [27]. In our study, both alterations correlated positively with NOS-2 expression by immunohistochemistry. Finally, the most common marker of renal damage, proteinuria correlated with NOS-2 expression, evaluated by three different methods.

The mechanisms involved in the regulation of COX-2 and TGF-β1 by NOS-2 are beyond the scope of the present study, nonetheless, several studies with different experimental models have shown that NO is

### Table 2. Correlations of NOS-2 expression obtained by different methods

<table>
<thead>
<tr>
<th>Correlation</th>
<th>R-value</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Gene analysis (RT–PCR)</td>
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</tr>
<tr>
<td>—NOS-2 (cortex) vs Uprot</td>
<td>0.77</td>
<td>0.001</td>
</tr>
<tr>
<td>—NOS-2 (cortex) vs COX2 (cortex)</td>
<td>0.77</td>
<td>0.001</td>
</tr>
<tr>
<td>—NOS-2 Cx vs TGF (cortex)</td>
<td>0.85</td>
<td>0.0001</td>
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<tr>
<td>Protein analysis (western blot and E)</td>
<td></td>
<td></td>
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<tr>
<td>—NOS-2_Cx,Mx vs Uprot</td>
<td>0.79</td>
<td>0.0005</td>
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<tr>
<td>—NOS-2_Cx,Mx vs COX2_Cx,Mx</td>
<td>0.84</td>
<td>&lt;0.0001</td>
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<tr>
<td>—NOS-2_Cx,Mx vs TGF_Elisa</td>
<td>0.87</td>
<td>&lt;0.0001</td>
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<tr>
<td>Immunohistochemical analysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>—NOS-2 vs Uprot</td>
<td>0.64</td>
<td>0.01</td>
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<tr>
<td>—NOS-2 vs M/L ratio</td>
<td>0.91</td>
<td>&lt;0.0001</td>
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<tr>
<td>—NOS-2 vs Fibrosis</td>
<td>0.88</td>
<td>&lt;0.0001</td>
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</table>
able to activate COX-2 [11] and TGF-β1 [12]. The increased expression of NOS-2, observed in this study, could be associated with an enhanced generation of NO or of a secondary cytotoxic product such as peroxynitrite, which has been shown to activate the latent fraction of TGF-β1 by inducing structural changes in its latency active peptide [12]. Thus, the active fraction of TGF-β1 may increase the deposition of extracellular matrix in renal interstitium, enhancing the fibrotic process.

An important finding of this study is that two selective inhibitors of NOS-2 such as AG and L-NIL showed a protective effect on microvascular lesions, although it was unable to prevent the rise of arterial pressure. We previously reported [4] that preservation of the microvascular structure prevents the glomerular hyperfiltration and hypertension that result from 5/6 Nx, despite persistence of arterial hypertension. Hence, we could speculate that conservation of the glomerular haemodynamic function, as a result of preservation of vascular structure, may be an additional protective effect exerted by AG and L-NIL.

In conclusion, the present studies show that the protective effect of two different selective inhibitors of NOS-2 (L-NIL and AG), inhibiting NOS-2 is associated with a decreased expression of COX-2, COX-2 and the active fraction of TGF-β1, as well as lower proteinuria and tubulointerstitial fibrosis, and the prevention of microvascular damage. These findings suggest that high concentrations of NO produced by NOS-2 can act as a positive modulator of proinflammatory and profibrotic pathways involved in the progression of renal disease.

Acknowledgements. We thank Dr José Pedraza-Chaverri for his suggestions and helpful discussions, and to Benito Chávez-Rentería for his technical assistance in the histological processing.

Part of this work was presented in the XV Scientific Meeting of the Inter-American Society of Hypertension. April 27–30, 2003, San Antonio, TX, USA.

Supported in part by grant CONACyT-Salud-2003-C01-25.

Conflict of interest statement. None declared.

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Received for publication: 7.6.06
Accepted in revised form: 27.6.06