Inducible Nitric Oxide Synthase–Derived Nitric Oxide Promotes Glomerular Angiogenesis via Upregulation of Vascular Endothelial Growth Factor Receptors

Abstract. The vascular endothelial growth factor (VEGF) system is of major importance for glomerular endothelial repair in glomerulonephritis (GN) and is significantly affected by nitric oxide (NO) release. For investigating whether glomerular upregulation of inducible NO synthase (iNOS) in GN might affect VEGF-mediated repair, a selective iNOS inhibitor, L-N6-(1-iminoethyl)-lysine (L-NIL), was administered to rats with anti-Thy 1.1 GN from day −2 until day 5 after GN induction. Compared with untreated nephritic rats, L-NIL–treated nephritic rats showed similar mean arterial BP, significantly decreased de novo peak nitrate production, and increased albuminuria on day 6. This was preceded by a significant decrease of glomerular endothelial cell proliferation and endothelial area on day 2 in L-NIL–treated nephritic rats. Uregulation of glomerular VEGF mRNA and protein expression, in particular of the VEGF164 splicing variant, occurred similarly in L-NIL–treated and untreated nephritic rats on days 2 and 7. However, the upregulation of glomerular VEGF receptor 1 and 2 mRNA expression on day 2 was reduced by 77 and 67%, respectively, in L-NIL–treated nephritic rats as compared with untreated nephritic rats. In parallel, glomerular VEGF165 binding was reduced by 34% in L-NIL–treated nephritic rats on day 2. Glomerular upregulation of the VEGF165 co-receptor neuropilin-1 mRNA in nephritic rats was reduced by L-NIL treatment only on day 7. Healthy untreated or L-NIL–treated controls showed no significant differences in any parameter analyzed. In conclusion, impaired repair of glomerular endothelium and downregulation of glomerular VEGF receptor expression was observed after selective iNOS inhibition in experimental GN. These data identify iNOS-derived NO production as the first in vivo regulator of the glomerular VEGF system and as an important mechanism promoting glomerular healing.

Understanding the regulation of glomerular capillary repair, i.e., angiogenesis, is of central importance in designing novel therapeutic approaches to glomerular diseases characterized by endothelial damage such as thrombotic microangiopathy, pre-eclampsia, or transplant glomerulopathy but is potentially also relevant for any disease that leads to glomerulosclerosis (1). In experimental situations, such as anti-Thy 1.1 mesangioproliferative glomerulonephritis (GN) in rats, glomerular capillary repair with features of angiogenesis is an integral part of the healing process (2,3), rendering this model particularly useful to identify mediators involved in glomerular capillary repair.

Of the various factors that regulate angiogenesis, vascular endothelial growth factor-A (VEGF-A; often used synonymously for VEGF) is a highly attractive candidate given that specific antagonism of VEGF165 or administration of VEGF165 augment or diminish, respectively, glomerular endothelial damage in the anti-Thy 1.1 GN model (4,5). Data on the glomerular expression of the various components of the VEGF system in rats with anti-Thy 1.1 GN are fragmentary. Glomerular VEGF release was augmented in the mesangioproliferative phase of anti-Thy 1.1 GN (6) and in a similar model, nephritis induced by antithymocyte serum, upregulation of glomerular VEGF- and VEGF receptor-2 mRNA was noted (2). No information is available on the expression of neuropilin, a VEGF co-receptor, in this model.

Nothing is currently known on the factors that regulate VEGF and VEGF receptor expression in vivo in instances of ongoing glomerular angiogenesis. In vitro, VEGF expression is increased by high glucose and TGF-β in cultured podocytes and by TGF-β in cultured mesangial cells (7,8), whereas aberrantly glycosylated IgA downregulated it in human mesangial cells (9). In mesangial cell culture, nitric oxide (NO) was identified as a rapid yet transient inducer of VEGF production (10). In addition, NO has been shown to downregulate and PDGF-BB as well as fibroblast growth factor-2 to upregulate the expression of VEGF-R1 mRNA in mesangial cells (10).

Whereas glomerular NO is normally generated by the two
constitutively expressed NO synthases (NOS; endothelial and neuronal NOS) (11), in glomerular damage, including the anti-Thy 1.1 GN model, a major increase in NO production has been linked to upregulation of the inducible NOS (iNOS) (12–14). The primary source of iNOS in the early phase of the anti-Thy 1.1 GN seems to be monocytes/macrophages and infiltrating neutrophils (13,15,16). To investigate the role of glomerular NO release in glomerular disease, previous studies made use of NG-nitro-l-arginine methyl ester (l-NAME) or NG-nitro-l-arginine methyl ester (l-NAME), i.e., unselective inhibitors of all three NOS isoforms. No clear message evolved from these studies, as these compounds improved the course of experimental lupus nephritis and anti-Thy 1.1 GN (17,18) but aggravated that of thrombotic microangiopathy and nephrotic serum nephritis (19,20). Because glomerular iNOS is specifically and prominently upregulated in anti-Thy 1.1 GN, we recently used the selective iNOS inhibitor l-NAME (l-NAME) in this model. Contrary to our expectations, specific iNOS inhibition aggravated the disease (21).

Given the above, the present study served two purposes: first, to characterize better the changes of the glomerular VEGF system in anti-Thy 1.1 GN and, second, to investigate the potential relationship between iNOS-derived NO and glomerular angiogenesis. We identify upregulated NO production as the first in vivo modulator of glomerular VEGF activity and demonstrate that glomerular angiogenesis is regulated through both iNOS-independent NO production and NO-dependent VEGF receptor overexpression.

Materials and Methods
Selective Inhibition of iNOS
Inhibition of iNOS was achieved using l-NIL, which has been described in detail previously (22). Briefly, l-NIL belongs to the acetylimidazol-containing analogues of arginin. It is 23-fold more selective for iNOS versus nNOS and 49-fold more selective for iNOS versus eNOS. It has no other known pharmacologic actions apart from competition with l-arginine for cellular uptake, and it has been used widely to probe the effects of iNOS inhibition (21–24). The in vivo dose of l-NIL (60 mg/kg) was chosen to resemble directly a previous study in the same model for reasons of comparability (21).

Experimental Model and Experimental Design
All animal studies were approved by the Institutional Review Board. Twenty-eight 8- to 9-wk-old LEW/Maa rats were purchased from the vivarium Centrale Proefdier Voorziening (CPV) of the University of Limburg (Maastrict, The Netherlands). Anti-Thy 1.1 mesangial proliferative GN was induced in 20 of these rats by a single intravenous bolus injection of a monoclonal anti-Thy 1.1 antibody (ER4-hybridoma; 1 mg/kg body wt). Eight control rats received an injection of PBS (pH 7.4) instead. The body weights (220 g at the beginning of the experiment and 260 g at day 7 after disease induction) were not significantly different between all treatment groups at any time point. Selective iNOS inhibition in 10 nephritic and four healthy control rats was achieved by administration of 60 mg/kg body wt l-NIL per day in the drinking water. The dosage was chosen on the basis of previous experience (21) and assuming that fluid intake compares well to urine volumes. Rats were housed in metabolic cages to measure urine output (range, 14 to 20 ml/d), and concentrations of l-NIL in the drinking water were prepared accordingly. The residual 10 nephritic and four healthy rats received tap water. Treatment was started 2 d before disease induction and continued until day 5, because glomerular NO production in untreated nephritic rats with anti-Thy 1.1 nephritis decreases to baseline at this time point (15). Twenty-four-hour urine collections were performed before starting the experiment, from days 1 to 2 and from days 6 to 7. The rats received an intraperitoneal injection of the thymidine analogue 5-bromo-2-deoxyuridine (BrdU; 100 mg/kg; Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) 4 h before they were killed. Nephritics animals were killed under ether anesthesia on day 2 (l-NIL–treated nephritic, n = 5; untreated nephritic, n = 5) and day 7 (l-NIL–treated nephritic, n = 5; untreated nephritic, n = 5). Healthy controls were killed together with the second nephritic group on day 7 (l-NIL–treated healthy, n = 4; untreated healthy, n = 4). Kidneys were perfused with 25 ml of ice-cold PBS and removed, and a renal cortical section per rat was obtained for light microscopy. The remaining cortical tissue of each animal was used to generate a preparation of glomeruli by differential sieving (14,21). All glomerular isolates were checked microscopically and exhibited a purity of >95%. The isolated glomeruli were used for the preparation of protein lysates, for the isolation of RNA, and the remainder was finally pooled for ex vivo culture of glomeruli to ensure a concentration sufficient to detect changes in NO2/NO3 secretion into the supernatants (see below).

Nitrate and Nitrite Measurements in Culture Supernatants and Urine
Isolated glomeruli were cultured at a concentration of 3000/ml in phenol red–free DMEM supplemented with penicillin/streptomycin, l-glutamine, tetrahydrobiopterin, 0.5% FBS, and 2 μg/ml LPS (Scerotype 0127:B8; Sigma Aldrich). After 48 h, supernatants were collected and centrifuged at 4000 rpm to remove glomeruli. Twenty-four-hour urine was collected using metabolic cages followed by centrifugation at 5200 × g for 10 min to remove food and cellular debris. For the measurements, urine samples were diluted 30-fold in water.

All samples were treated with nitrate reductase (from Aspergillus species, 0.1 U/100 μl; Boehringer Mannheim, Mannheim, Germany) in the presence of NADPH before nitrite measurements using the Griess assay as described previously (15). Colorimetric reaction was determined using an automated plate reader (Dynatech) reading extinction at 550 nm and compared with a standard curve of sodium nitrate.

Renal Morphology
Tissue for light microscopy and immunoperoxidase staining was fixed in methyl Carnoy’s solution and embedded in paraﬃn. Four-micrometer sections were stained with the periodic acid-Schiff reagent and counterstained with hematoxylin. In periodic acid-Schiff–stained sections, the number of mitoses as well as the grade of mesangiolysis within 50 to 100 glomerular tufts was determined. Using a 1000-fold magnification, mitoses were differentiated into those that clearly localized to a glomerular capillary lumen and were inside the glomerular basement membrane, subsequently referred to as proliferating endothelial cells, and mitoses in any other localization within the glomerular tuft. Mesangiolysis was graded on a semiquantitative scale as 0 = no mesangiolysis, 1 = segmental mesangiolysis, II = global mesangiolysis, and III = microaneurysm, as described (25).

Immunoperoxidase Staining
Four-micrometer sections of methyl Carnoy’s ﬁxed biopsy tissue were processed by an indirect immunoperoxidase technique as de-
scribed previously (26). To detect glomerular endothelial cells, we used JG-12, a monoclonal antibody to rat endothelial cells. Specificity of this antibody for rat renal microvascular endothelial cells was reported in detail recently (27). Kang et al. (28) compared the microvascular staining pattern using the JG-12 and RECA-1 antibodies, which detect different endothelial antigens, and noted high similarity of the data. This supports relatively stable, constitutive antigen expression on endothelial cells. To detect glomerular proliferating cells that incorporated BrdU into the nucleus, we used BU-1, a mouse monoclonal antibody to BrdU (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany). Negative controls consisted of substitution of the primary antibody with equivalent concentrations of mouse IgG. The sections were then incubated with biotinylated horse anti-mouse antibody (Vector Labs, Burlingame, CA). The ABC-Elite reagent (Vector Labs) and finally 3,3′-diaminobenzidine (with nickel chloride enhancement) were used as the chromogen. Sections were counterstained with methyl green. All slides were evaluated by an observer, who was unaware of the origin of the slides.

The immunostaining for JG-12 was evaluated using a point-counting method. For this, a grid composed of 100 dots was superimposed on consecutive glomeruli (range, 25 to 30; magnification, 1000-fold), and the percentages of dots overlying stained areas were counted (27).

**VEGF Protein Analysis in Glomerular Lysates**

Isolated glomeruli were homogenized in 2 ml of Triton X-100 lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM PMSF) at 4°C. After incubation for 5 min, lysates were centrifuged at 4°C for 15 min at 10,000 × g. The protein concentrations were determined by the method of Lowry et al. (29). VEGF protein in the lysates was measured using an ELISA: 96-well MaxiSorp plates (Nunc GmbH & Co KG, Wiesbaden, Germany) were coated overnight at 4°C with a polyclonal goat anti-VEGF antibody (293 NA, R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany), which was diluted 1:500, for 1 h at room temperature, and two washing steps followed. The detecting antibody was polyclonal rabbit anti-VEGF IgG (Genzyme Virotech GmbH, Rüsselsheim, Germany), 1:500, for 1 h at room temperature, followed by three washing steps. Incubation with biotinylated anti-rabbit IgG (Vector Labs; 1:400, 1 h room temperature) was followed by four washing steps, incubation with streptavidin-coupled horseradish peroxidase (Vector Labs, 1:1000, 1 h room temperature), five washing steps, and incubation with the peroxidase substrate tetramethylbenzidine (100 μg/ml in acetate/citrate buffer [pH 4.9], 10 min at room temperature in the dark). The reaction was stopped with 2 N H₂SO₄, and colorimetric reaction was determined using an automated plate reader (Dynatech Deutschland GmbH, Denkendorf, Germany), reading extinction at 450 nm. All measurements were performed in duplicate.

**Reverse Transcriptase–PCR**

Differential reverse transcriptase–PCR (RT-PCR) was performed as described previously (30). Briefly, total RNA was extracted from the isolated rat glomeruli with the guanidinium isothiocyanate/phenol/chloroform method using standard procedures. The RNA content and the purity of the samples obtained was measured by UV spectrophotometry at 260 and 280 nm with OD₂₆₀/₂₈₀ ratios of ~2.0, demonstrating a clean RNA. cDNA was synthesized from 80 ng total RNA with the Moloney murine leukemia virus reverse transcriptase (Life Technologies, Karlsruhe, Germany) according to the manufacturer's instruction. The quality of the cDNA was confirmed by amplification of cDNA other than VEGFR-cDNA and VEGF cDNA (see below), e.g., of GAPDH, PDGF-A, and PDGF-B without restrictions. Amplified cDNA fragments and primers are shown in Table 1. Simultaneous amplification of the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) was included in each PCR reaction as an internal control (Table 1).

Differential quantitative PCR is sensitive to the number of cycles and the quantity of primers. Before analyzing the glomerular RNA of the treated animals, we therefore optimized cycle numbers and primer quantity for the different amplification reactions. The PCR optimization study was done starting with 80 ng total of glomerular RNA of a nephritic day 2 animal and 80 ng total of glomerular RNA of a nephritic day 7 animal, resulting in identical optimal PCR conditions. These conditions in a 50-μl reaction (amplification reaction is loga-

### Table 1. Primers and PCR conditionsa

<table>
<thead>
<tr>
<th>mRNA Species</th>
<th>Primer Sequences</th>
<th>No. of PCR Cycles and Quantity of Primers (50-μl Reaction Mix)</th>
<th>Quantity of Primers of Housekeeping Gene HPRT (50-μl Reaction Mix)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat VEGF-R1 (396-bp fragment)</td>
<td>5′-cggatctacgagcagcaagttac-3′</td>
<td>28 cycles</td>
<td>60 pmol</td>
</tr>
<tr>
<td>Rat VEGF-R2 (523-bp fragment)</td>
<td>5′-gctggctgactagctgctgctgct-3′</td>
<td>40 pmol</td>
<td></td>
</tr>
<tr>
<td>Rat VEGF (exons 1–5) (101-bp fragment)</td>
<td>5′-gggagcctggtctgtagcagag-3′</td>
<td>26 cycles</td>
<td></td>
</tr>
<tr>
<td>Rat neuropilin-1 (310-bp fragment)</td>
<td>5′-tcctcctagcctgctgctgctgct-3′</td>
<td>80 pmol</td>
<td></td>
</tr>
<tr>
<td>Rat HPRT (231-bp fragment)</td>
<td>5′-tagctgcagcagctgctgctgctgct-3′</td>
<td>60 pmol</td>
<td></td>
</tr>
<tr>
<td>Rat VEGF splice variants</td>
<td>5′-tcacggtgctgctgctgctgctgctgct-3′</td>
<td>35 cycles</td>
<td></td>
</tr>
</tbody>
</table>

a HPRT, hypoxanthine-guanine phosphoribosyltransferase; VEGF, vascular endothelial growth factor.
rithmic and independent of the quantity of primers) are shown in Table 1. The PCR reactions were performed with Taq Polymerase (Roche, Mannheim, Germany), 1.5 mM MgCl₂, and 10 mM dNTP Mix (Amersham Pharmacia, Freiburg, Germany). Reaction conditions were 5 min at 94°C followed by cycles of 40 sec at 94°C, 40 sec at 62°C, and 40 sec at 72°C. The samples were subsequently electrophoresed in a 1.5% agarose gel, and the bands were quantified by densitometry using the system of Biostep GmbH (Jahnsdorf, Germany). Specific band intensities were scanned and corrected for the relative intensities of the HPRT band. The different numbers of required PCR cycles (Table 1) can account for the observed differences of HPRT band intensities between Figures 1 through 3. All measurements were performed in triplicate. The analysis software was from TotalLab (Phoretix International, Newcastle, UK).

For the detection of the rat VEGF splicing variants VEGF₁₄₈, VEGF₁₆₅, VEGF₁₄₄, and VEGF₁₂₁, primers spanning the start of exon 1 to the end of exon 8 were used (Table 1). PCR reactions were performed with Taq DNA polymerase (Roche), 1.5 mM MgCl₂, 1 mM primers, and 10 mM dNTP mix (Amersham Pharmacia). PCR conditions were 5 min at 94°C followed by 35 cycles for 1 min at 94°C, 58°C, and 72°C, respectively, with an 8-min extension time at 72°C on cycle 35. Amplified splicing variants were separated on a 1.5% agarose gel, and single bands were excised, subcloned in pBluescript II (Novagen, Bad Soden, Germany), and sequenced. These clones were used as controls for the analyses of VEGF splicing variants in the glomerular RNA of L-NIL–treated or untreated nephritic and normal rats. Amplified bands were analyzed on a 1.5% agarose gel, and the relative amount of each band was quantified by densitometry as described above. The measurements were performed in triplicate. Finally, the ratio between the relative amount of each glomerular VEGF variant in each animal to the respective amount of the smallest VEGF splicing variant, namely VEGF₁₁₀, was calculated.

**VEGF₁₆₅ In Situ Receptor Binding**

Binding experiments were performed on 10-μm frozen tissue sections by incubation with ¹²⁵I-VEGF₁₆₅. Sections were preincubated at room temperature for 30 min in DMEM supplemented with 10% FCS, 25 mM HEPES (pH 7.4), 0.5 mM MgCl₂, 4 μM leupeptin, and 5 nM PMSF. The preincubation buffer was removed, and the sections were covered by a drop of the same buffer that contained 40 pM human ¹²⁵I-VEGF₁₆₅ (Perkin Elmer Life Sciences, Zaventem, Belgium). The homology between human and rat VEGF is >89% in the VEGF receptor binding domain (amino acids 8 to 109 of the mature protein), and specific as well as efficient binding of human ¹²⁵I-VEGF₁₆₅ to rat tissue was previously demonstrated (31). Nonspecific binding was determined on adjacent sections incubated with the same concentration of ¹²⁵I-VEGF₁₆₅ in the presence of 25-fold excess of unlabeled VEGF. After a 4-h incubation, the slides were washed twice in PBS and once in 1.5 M NaCl to remove nonspecific binding to proteoglycans and fixed and air-dried.

After being dipped in NTB2 nuclear emulsion (Kodak, Rochester, NY), slides were exposed in the dark at 4°C for 2 wk. After development, slides were counterstained with hematoxylin and eosin, dehydrated, and coverslipped with Histokitt (Rot, Karlsruhe, Germany). Microscopy of the sections included bright and dark field illumination.

**Miscellaneous Measurements.**

Urinary albumin levels were determined on a 96-well ELISA plate, using a peroxidase-conjugated anti-rat albumin antibody (ICN-Biomedical, Eschwege, Germany), as described (21). All measurements were performed in duplicate. BP measurements were performed by the tail-cuff method, using a programmed sphygmomanometer, BP-98A (Softron, Tokyo, Japan).

**Statistical Analyses**

All values are expressed as means ± SD. Statistical significance (defined as $P < 0.05$) between L-NIL–treated and untreated animals were performed in duplicate. BP measurements were performed by the tail-cuff method, using a programmed sphygmomanometer, BP-98A (Softron, Tokyo, Japan).
was evaluated with the unpaired t test. When more than two groups were compared, analysis was done by ANOVA with the Bonferroni correction for multiple comparisons.

Results

Glomerular VEGF and VEGF Receptor Expression Is Upregulated in Anti-Thy 1.1 GN

VEGF mRNA and Protein. Using a primer pair, which amplifies a sequence common to all rat VEGF isoforms, on day 2 after disease induction, glomerular VEGF mRNA content increased 1.7-fold over normal glomeruli, but this increase failed to reach statistical significance (Table 2). Glomerular VEGF protein levels increased significantly from concentrations below the detection threshold of the assay to ~1 pg/μg
total protein on day 2 (Table 2). mRNA levels returned to normal or even decreased on day 7 of the disease, whereas VEGF protein levels fell but remained significantly elevated in comparison with nonnephritic rats (Table 2).

**VEGF Splicing Variants.** Next, we asked whether changes in the relative amount of single alternatively spliced VEGF isoforms occurred in nephritic glomeruli. For this, RT-PCR was performed with a primer pair amplifying VEGF cDNA between exon 1 and exon 8 (Figure 4). Four glomerular VEGF splicing variants, namely VEGF_{188}, VEGF_{164}, VEGF_{144}, and VEGF_{120}, with VEGF_{164} being the most prominent one, were detected (Figure 4A). By densitometry, we assessed the relationship of each splicing variant to the smallest one (VEGF_{120}). The data (Figure 4B) showed that the splicing pattern was not significantly altered in the nephritic animals on days 2 and 7 after disease induction in comparison with normal controls.

**VEGF Receptors.** In addition to VEGF protein, regulation of glomerular angiogenesis might involve changes in VEGF receptor expression. Differential quantitative RT-PCR of the main receptors VEGF-R1 and -R2 showed a pronounced and very transient increase of receptor transcripts in the nephritic rats on day 2 after disease induction as compared with the normal controls, whereas on day 7, the transcript expression fell to near-normal levels (Figures 1 and 2). Quantitative RT-PCR for the VEGF_{164} co-receptor neuropilin-1 also showed an increased transcript expression in the nephritic rats on day 2, which was diminished on day 7 but still increased compared with the normal controls (Figure 3). Because of lack of specificity and/or sensitivity of available VEGF receptor antibodies, glomerular VEGF receptor protein expression could not be studied by Western blot analysis or immunohistochemistry. We therefore chose an indirect assessment by studying glomerular binding of labeled VEGF to the renal sections. Compared with the normal controls (Figure 5A), nephritic rats (Figure 5C) exhibited a significant 2.2-fold increase of glomerular VEGF binding sites on day 2 (Figure 5H). Increased VEGF-binding was efficiently blocked by incubation of these tissues with labeled VEGF together with a 25-fold excess of unlabeled VEGF (Figure 5G). On day 7 after disease induction, however, the numbers of glomerular VEGF binding sites were similar to those of the normal controls (Figure 5, E and H).

### Table 2. Glomerular VEGF mRNA and protein levels

<table>
<thead>
<tr>
<th>Groups</th>
<th>Glomerular VEGF mRNA (Arbitrary Units) Day 2 after Disease Induction</th>
<th>Glomerular VEGF mRNA (Arbitrary Units) Day 7 after Disease Induction</th>
<th>Glomerular VEGF Protein (pg/μg Total Protein) Day 2 after Disease Induction</th>
<th>Glomerular VEGF Protein (pg/μg Total Protein) Day 7 after Disease Induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nephritic untreated</td>
<td>1.47 ± 1.08</td>
<td>0.38 ± 0.29</td>
<td>1.19 ± 0.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.64 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nephritic + L-NIL 60 mg/kg daily (days −2 to 5 after disease induction)</td>
<td>1.93 ± 1.43</td>
<td>0.84 ± 0.62</td>
<td>1.00 ± 0.32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.44 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Normal untreated</td>
<td>0.86 ± 0.17</td>
<td>&lt;0.28</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Normal + L-NIL 60 mg/kg daily (7 d)</td>
<td>1.02 ± 0.30</td>
<td>&lt;0.15</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data are mean values ± SD; n = 5 for each nephritic group, and n = 4 for each normal group. L-NIL, L-N6-(1-iminoethyl)-lysine.
<sup>b</sup> P < 0.05 versus untreated and L-NIL–treated normal groups.

**Inhibition of iNOS Augments Albuminuria in Rats with Anti–Thy 1.1 GN**

To investigate the role of iNOS-derived NO in glomerular angiogenesis, we studied additional groups of rats that were...
treated with the specific iNOS inhibitor L-NIL from days −2 to 5 after disease induction. Using these rats, we first aimed to confirm our previous data (21) that iNOS inhibition can be achieved in anti-Thy 1.1 GN and that it aggravates the course of the disease.

**iNOS Inhibition Normalizes Renal NO Production in Nephritic Rats**

Urinary NO₂/NO₃ excretion (metabolites of NO) was markedly enhanced in nephritic rats on days 2 and 7 after disease induction as compared with controls (Table 3). Treatment of nephritic animals with L-NIL normalized the urinary NO₂/NO₃ excretion on both days (Table 3). Basal urinary NO₂/NO₃ excretion in nephritic animals remained unchanged during L-NIL treatment. Glomerular NO₂/NO₃ production was determined after 48-h culture of pooled glomeruli isolated on day 7 after disease induction. Again, a decreased NO₂/NO₃ release was measured in the L-NIL–treated nephritic group compared with the untreated nephritic group (Table 3). The level of NO₂/NO₃ released by cultured glomeruli isolated from the healthy control animals was below the detection limit. iNOS inhibition did not alter systemic normotension on day 5 after disease induction (Table 3), suggesting that L-NIL did not affect endothelium-dependent NO release. In addition, there was no influence of L-NIL treatment on body weights or on water intake (data not shown).

**iNOS Inhibition Aggravates Albuminuria in Anti-Thy 1.1 Nephritis**

Albuminuria was markedly increased in the nephritic animals compared with nonnephritic rats on day 7 after disease induction (Table 3). In confirmation of our previous findings (21), treatment of nephritic rats with L-NIL led to a significant increase of albuminuria, whereas basal albuminuria in nonnephritic controls remained unchanged by L-NIL treatment (Table 3). However, in contrast to our previous study (21), increased albuminuria in L-NIL–treated nephritic rats was not associated with an aggravation of mesangiolysis, glomerular microaneurysm formation, or evidence of increased intraglomerular thromboses (data not shown).
Because iNOS inhibition was started before the induction of anti-Thy 1.1 nephritis, one potential concern is that this treatment modified the glomerular binding of ER4 anti-Thy 1.1 antibody and thereby affected the induction phase of the disease. Two observations in previous studies argue against this possibility: (1) we noted no effect on the disease induction even with L-NMMA, which, unlike L-NIL, altered systemic hemodynamics and induced hypertension (18); and (2) early markers of glomerular damage, such as albuminuria and mesangiolysis on days 1 and 3, were not affected by L-NIL treatment (21).

**Inhibition of iNOS Aggravates Glomerular Endothelial Cell Damage in Rats with Anti-Thy 1.1 GN**

Glomerular endothelial cell turnover is maximal on day 2 after disease induction in anti-Thy 1.1 GN (4). Therefore, glomerular cell proliferation was analyzed at this time point in the different groups by counting both glomerular mitotic figures and BrdU-positive cells. A 43% (mitotic figures) to 67% (BrdU) decrease of glomerular cell proliferation was noted on day 2 in the L-NIL-treated nephritic animals compared with untreated nephritic rats (11.3 ± 3.0 versus 19.8 ± 3.6 mitotic figures per 100 glomeruli, respectively, \( n = 5, P < 0.05 \); and 0.56 ± 0.51 versus 1.71 ± 0.60 glomerular BrdU-positive cells per glomerulus, respectively, \( n = 5, P < 0.05 \)). We also assessed glomerular endothelial versus nonendothelial cell proliferation separately as described previously (4,27). As shown in Figure 6, iNOS inhibition in nephritic rats caused a significant decrease of glomerular endothelial cell proliferation on day 2 (60% reduction of glomerular endothelial mitoses and 82% reduction of glomerular endothelial nuclear BrdU incorporation) compared with the untreated nephritic animals. In contrast, proliferation of nonendothelial cells within the glomerular tuft did not differ significantly between L-NIL-treated and untreated nephritic rats on day 2 (Figure 6). The total number of glomerular mitotic figures on day 7 was not signif-

### Table 3. Urinary and glomerular NO\(_2/\)NO\(_3\) excretion, mean arterial blood pressure, and albuminuria\(^a\)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Urinary NO(_2/)NO(_3) Excretion (µmol NO(_2/d)) Day 2 after Disease Induction</th>
<th>Urinary NO(_2/)NO(_3) Excretion (µmol NO(_2/d)) Day 7 after Disease Induction</th>
<th>Glomerular NO(_2/)NO(_3) Production (µmol NO(_2/L)) Day 7 after Disease Induction and 48-h Culture(^b)</th>
<th>Mean Arterial Blood Pressure (mmHg) Day 5 after Disease Induction</th>
<th>Albuminuria (mg/24 h) Day 7 after Disease Induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nephritic + L-NIL 60 mg/kg daily (days -2 to 5 after disease induction)</td>
<td>16.4 ± 2.8(^c)</td>
<td>18.4 ± 2.0(^c)</td>
<td>117.9</td>
<td>96 ± 8</td>
<td>125 ± 59(^c)</td>
</tr>
<tr>
<td>Nephritic untreated</td>
<td>23 ± 2.5</td>
<td>27.4 ± 4.0</td>
<td>144.2</td>
<td>b.d.</td>
<td>61 ± 18</td>
</tr>
<tr>
<td>Healthy + L-NIL 60 mg/kg daily (7 d)</td>
<td>19.6 ± 2.7(^c)</td>
<td>b.d.</td>
<td>0.18 ± 0.11(^c)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy untreated</td>
<td>19.7 ± 3.0(^c)</td>
<td>b.d.</td>
<td>0.24 ± 0.16(^c)</td>
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</tbody>
</table>

\(^a\) Data are mean values ± SD; \( n = 5 \) for each nephritic group and \( n = 4 \) for each healthy group. Glomerular NO\(_2/\)NO\(_3\) production was assessed after 48-h culture of pooled glomeruli from all animals in each group. b.d., below detection limit.

\(^b\) Values are means of triplicate determination of pooled glomeruli.

\(^c\) \( P < 0.05 \) versus untreated nephritic treatment group.

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**Figure 6.** Glomerular endothelial versus nonendothelial cell proliferation in L-NIL-treated and untreated nephritic animals on day 2 after disease induction. Evaluation was carried out by counting glomerular 5-bromo-2'-deoxyuridine-positive nuclei as well as glomerular mitotic figures; \( n = 5 \) for each group.
significantly different between both nephritic groups (11.2 ± 5.8 mitoses per 100 glomeruli in the untreated group versus 13.7 ± 3.0 in the t-NIL–treated group). Most of the proliferating cells in both groups were nonendothelial cells (8.7 ± 5.0 nonendothelial mitoses per 100 glomeruli in the untreated versus 11.9 ± 1.7 in the t-NIL–treated animals compared with 2.5 ± 2.4 endothelial mitotic figures per 100 glomeruli in the untreated versus 1.8 ± 1.4 in the t-NIL–treated animals), and t-NIL had no significant influence on the number of endothelial mitoses at that late time point.

The effects of iNOS inhibition on glomerular endothelial cell turnover were studied further by immunostaining with an antibody specific for rat endothelium (Figure 7). Glomerular endothelial rarefaction was noted in nephritic animals on day 2 after disease induction compared with nonnephritic rats (Figure 7, A through C). t-NIL treatment of nephritic animals resulted in an augmented rarefaction of the glomerular endothelium, which was found to be highly significant compared with the untreated nephritic group (Figure 7D). In contrast, glomerular JG-12 staining in renal tissue of the nephritic day 7 rats (27.7 ± 6.0% stained glomerular area in untreated versus 28.5 ± 5.6% in t-NIL–treated rats) and of nonnephritic rats (Figure 7D) was not affected by t-NIL treatment.

Effects of iNOS Inhibition on the Glomerular VEGF System

After demonstrating that specific iNOS inhibition in anti-Thy 1.1 GN impairs glomerular capillary repair, we asked

![Figure 7. Effects of selective inducible nitric oxide synthase inhibition on glomerular endothelial cells. Renal glomerular endothelial cell staining with JG-12 in a normal rat treated with t-NIL (A), on day 2 in an untreated nephritic rat (B), and on day 2 in an t-NIL–treated nephritic rat (C). Glomerular endothelial cell staining in nephritic animals was decreased compared with normal rats and particularly decreased in t-NIL–treated nephritic animals compared with untreated nephritic animals. (D) Quantitative evaluation of glomerular JG-12 immunostaining in healthy controls and on day 2 after disease induction in nephritic animals by grid counting; n = 4 for each normal group, and n = 5 for each nephritic group. Magnification, ×600.](image-url)
whether this effect was paralleled by changes in the glomerular VEGF system.

**VEGF mRNA and Protein.** As shown in Table 2, neither glomerular VEGF transcript expression nor VEGF protein expression differed significantly between l-NIL–treated and untreated nephritic animals on day 2 as well as on day 7 after disease induction.

**VEGF Splicing Variants.** Inhibition of iNOS in anti–Thy 1.1 GN also had no effect on the relative abundance of any of the VEGF splicing variants in the nephritic animals on day 2 as well as on day 7 after disease induction.

**VEGF Receptors.** As shown in Figures 1 and 2, the increase of VEGF-R1 and -R2 in nephritic glomeruli on day 2 was markedly decreased in the l-NIL–treated nephritic group as compared with the untreated nephritic group: VEGF-R1 transcript expression decreased by 77%, and VEGF-R2 mRNA decreased by 67%. In part, this might be due to the rarefaction of glomerular endothelial cells at this time point in the l-NIL–treated animals. However, most likely, this cannot be explained by rarefaction alone, because, as assessed by JG-12 immunostaining, endothelial cell surface decreased maximally by 15% (Figure 7). On day 7 after disease induction, both glomerular VEGF-R1 and -R2 transcripts in the l-NIL–treated animals were at the level of those in the healthy controls and showed no significant differences compared with the expression levels of the untreated nephritic animals. The increased neuropilin-1 transcript expression on day 2 in the nephritic animals was not changed but was reduced by 49% on day 7 by l-NIL treatment (Figure 3). Overexpression of neuropilin-1 on day 7 is most likely due to the massive increase in mesangial cell numbers. These cells have been demonstrated to express neuropilin-1 in vitro (32). Treatment of normal rats with l-NIL had no effect on VEGF receptor transcript expression. In parallel to these l-NIL–induced changes in VEGF receptor mRNA expression, glomerular VEGF<sub>165</sub> binding sites also decreased significantly by 34% on day 2 in nephritic animals that received l-NIL (Figure 5, D and H). The number of glomerular VEGF binding sites remained unchanged on day 7 in nephritic rats and also in normal rats that received l-NIL (Figure 5, F, A, B, and H, respectively).

**Discussion**

In this study, we aimed to dissect the effects of selective iNOS inhibition on the VEGF system and on endothelial cell repair in the model of anti–Thy 1.1 GN in LEW/Maa rats. The first major finding of our study was that early glomerular capillary injury in anti–Thy 1.1 GN led to a transient upregulation of VEGF protein expression. Whereas previous studies documenting glomerular VEGF overexpression in anti–Thy 1.1 GN (6) have focused on the late, mesangioproliferative phase, we demonstrate that the main VEGF increase occurred on day 2. This observation seems to be of particular importance, given that the wave of glomerular endothelial proliferation is an early event in anti–Thy 1.1 GN and has largely subsided in the later, mesangioproliferative phase (4). The relative abundance of VEGF splicing variants in the glomerulus did not change significantly during anti–Thy 1.1 GN, and the 164-isoform remained the dominant species, which is consistent with observations in isolated podocytes (33). In addition to podocyte-derived VEGF, glomerular VEGF expression can be induced in mesangial cells in vitro (10,34–37). More important, in vivo in human mesangioproliferative GN an apparent de novo expression of VEGF has been localized to mesangial regions as well (38). Similar to this, in the present study, an increased glomerular VEGF expression persisted in the mesangioproliferative phase of anti–Thy 1.1 GN, i.e., day 7.

In addition to glomerular VEGF, a major upregulation of glomerular VEGF receptor mRNA occurred very early in anti–Thy 1.1 GN. Expression of VEGF-R1 and -R2 mRNA in normal kidney has been localized to glomerular and peritubular capillaries as well as to pre- and postglomerular vessels (39–41), whereas neuropilin-1 expression in normal kidney has been localized to podocytes (42). In cultured mesangial cells, expression of VEGF-R1 and -R2 and neuropilin-1 has been demonstrated (10,32,43), and circulating monocytes have been identified to express VEGF-R1 but not -R2 as well (44–46). Consequently, the early increase of glomerular VEGF receptor mRNA content in anti–Thy 1.1 GN is particularly remarkable, given that on day 2 the mesangium is largely destroyed and considerable loss of endothelial cells occurs secondary to the mesangial damage. It is conceivable that bone marrow–derived endothelial progenitor cells (47) expressing VEGF receptors replaced lost endothelial cells, for example, mediated by Hypoxia Inducible Factor (HIF)-1α–induced systemic VEGF upregulation (48). However, in the anti–Thy 1.1 GN model, the existence of bone marrow–derived cells in endothelial locations is controversial (49,50). Apart from showing an increase in glomerular VEGF receptor mRNA, we aimed to confirm this increase at the protein level. Given the difficulty in demonstrating VEGF receptor protein expression by Western blotting or immunohistochemistry, we reverted to VEGF-binding studies, which confirmed an early increase in glomerular VEGF binding sites. One limitation of this approach is that it cannot distinguish between VEGF binding to the two VEGF receptors and the accessory receptor neuropilin-1, which enhances binding of VEGF<sub>165</sub> to VEGF-R2 (51,52).

The third major finding of the present study was that specific iNOS inhibition aggravated the course of anti–Thy 1.1 GN, as already noted previously (21). The new observation was that this effect was associated with a specific reduction of glomerular endothelial cell proliferation and restitution. In addition, in the present study, aggravation of glomerular damage by iNOS inhibition could be separated from increased glomerular coagulation or platelet localization, suggesting that in our previous study, endothelial damage might have been more pronounced and might have resulted in the loss of anticoagulant activity in the damaged glomeruli. In combination, however, our two studies’ data suggest that modulation of glomerular angiogenesis by iNOS–derived NO may be more central in explaining the increased glomerular damage than altered intraglomerular coagulation or even be a causative factor for enhancing local coagulation, respectively. Consistent with this interpretation is the fourth major finding, namely that l-NIL treatment specif-
ically reduced glomerular VEGF receptor mRNA expression and VEGF binding sites in early anti-Thy 1.1 GN, whereas it did not affect the glomerular overproduction of VEGF, which is confined to mesangial cells and/or podocytes (10,33–38). Our data do not allow us to dissect the question of whether reduced endothelial proliferation led to reduced VEGF receptor expression or vice versa. Taken together, however, these findings suggest that the effects of L-NIL were relatively specific for the glomerular endothelium. In support of this interpretation, we failed to observe an increase in mesangiolysis and microaneurysm formation, consequences that would be expected if mesangial repair is impaired, as has been noted, for example, after heparin administration (25) or PDGF-B inhibition (53) or unselective NOS blockade (18).

The interactions between NO and the VEGF system are complex. VEGF mediates a central part of its proangiogenic effects through stimulation of eNOS- and possibly iNOS-derived NO (54,55). Consequently, if NO can act as a downstream mediator of VEGF, then decreased angiogenesis may result from iNOS inhibition despite unchanged VEGF levels. This would be compatible with our result from iNOS inhibition despite unchanged VEGF levels. Consequently, if NO can act as a downstream mediator of VEGF, then decreased angiogenesis may result from iNOS inhibition despite unchanged VEGF levels.

Little is known of the relative roles of VEGF-R1 versus R2 in the glomerulus. It is generally assumed that the activity of VEGF is largely mediated through the VEGF-R2, whereas the role of VEGF-R1 is variably described as antagonistic, i.e., serving as a nonsignaling “decoy” receptor, or agonistic under some circumstances (52). In addition, VEGF-R1 may be responsible for the release of tissue-specific growth factors in a vascular bed–specific manner (52). Buusolati et al. (58) recently showed in an elegant in vitro study in human umbilical vein endothelial cells that VEGF-R1 is a signaling receptor that promotes endothelial cell differentiation into vascular tubes, in part by limiting VEGF-R2–mediated endothelial cell proliferation. This effect of VEGF-R1 on the VEGF-R2 seemed to be mediated by eNOS-derived NO. However, because in that study both VEGF receptors were inhibited separately, the net effect of inhibition or downregulation of both receptors remained unknown.

In summary, our study identifies upregulated NO production resulting from iNOS activity as the first in vivo modulator of glomerular VEGF activity and demonstrates that glomerular angiogenesis is regulated through iNOS-dependent VEGF receptor overexpression and iNOS-independent upregulation of VEGF synthesis. On the basis of these observations, it remains to be tested whether stimulation of iNOS or the administration of NO donors in instances of ongoing glomerular angiogenesis may represent a novel therapeutic approach to diseases such as thrombotic microangiopathy, preeclampsia, and transplant glomerulopathy.


