Autonomic Renal Denervation Ameliorates Experimental Glomerulonephritis

Roland Veelken,* Eva-Maria Vogel,* Karl Hilgers,* Kerstin Amann,† Andrea Hartner,‡ Gabriele Sass,§ Winfried Neuhuber,§ and Gisa Tiegs§

*Department of Nephrology and Hypertension, †Institute of Pathology, ‡Children and Youth Hospital, §Institute of Experimental and Clinical Pharmacology and Toxicology, and Institute of Anatomy I, University of Erlangen-Nuremberg, Erlangen, Germany

ABSTRACT

Increasing evidence indicates that inflammation of visceral organs is significantly affected by the autonomic nervous system. Such neuroimmune interactions have not been studied in the kidney. Here, we show that the rat kidney is innervated by both tyrosine hydroxylase–positive sympathetic efferent nerve fibers and calcitonin gene-related peptide–positive primary afferent nerve fibers, both of which are found in proximity to macrophages and dendritic cells. Complete surgical bilateral renal denervation was performed 2 d before glomerulonephritis was induced by injecting the monoclonal anti–Thy-1.1 antibody OX-7. Denervation significantly reduced albuminuria, mesangiolysis, formation of microaneurysms, deposition of glomerular collagen IV, and expression of TGF-β compared with sham-operated controls. Accordingly, inflammation, identified by accumulation of interstitial macrophages and renal expression of TNF-α, and mesangial cell proliferation were significantly reduced. These findings indicate that autonomic renal denervation ameliorates and, by inference, innervation exacerbates acute inflammation in the kidney; therefore, neurotransmitters or neuropeptides and their receptors might represent novel targets for the treatment of acute glomerulonephritis.


Increasing evidence supports a bidirectional interaction between the nervous and immune systems. This has been documented by anatomic studies showing innervation of primary and secondary lymphoid organs, as well as functional studies in acute and chronic inflammation of the respiratory and gastrointestinal tract, the skin, and joints. We recently reported that liver inflammation is also modulated by the autonomic nervous system. Sympathetic efferent as well as primary sensory neurons are key players in the regulation of peripheral inflammation and immune responses. Primary sensory neurons innervating inner organs are composed mainly of the peptidergic subpopulation of B-type primary afferents giving rise to unmyelinated C and thin myelinated Aδ fibers. They transmit nociceptive information to the central nervous system (afferent function), whereas their peripheral terminals release substance P (SP), calcitonin gene-related peptide (CGRP), and other neuropeptides during injury and inflammation (local effector function, neurogenic inflammation). Hence, catecholamines as well as peptide transmitters may contribute to neuroimmune modulations. In concert with β2 adrenergic receptor effect of catecholamines, the co-transmitter of sympathetic neurons neuropeptide Y as well as somatostatin.

Received May 9, 2007. Accepted February 14, 2008. Published online ahead of print. Publication date available at www.jasn.org.

R.V. and E.-M.V. contributed equally to this work

Correspondence: Prof. Gisa Tiegs, Institute of Experimental and Clinical Pharmacology and Toxicology, University of Erlangen-Nuremberg, Fahrstrasse 17, D-91054 Erlangen, Germany. Phone: +4991318522283; Fax: +4991318522774; E-mail: tiegs@ pharmakologie.uni-erlangen.de

Copyright © 2008 by the American Society of Nephrology
vasoactive intestinal peptide, and CGRP seem to down-modulate the inflammatory response. In contrast, norepinephrine, acting through \(\alpha_1\) and \(\alpha_2\) adrenergic receptors (predominant in chronic inflammation) as well as SP, exhibit proinflammatory properties. Proinflammatory cytokines such as TNF-\(\alpha\) and IL-1\(\beta\) can either directly reach the central nervous system via the circulation or alternatively stimulate peripheral afferent nerve fibers, thereby activating central neurons in specific brain areas and probably also autonomic efferent nerves. The bidirectional communication between the nervous and immune systems results from the expression of specific receptors for cytokines and other immune mediators on neurons, as well as for neurotransmitters and neuropeptides on immune cells.

Although the kidney is frequently affected by immune-mediated pathologies such as various forms of glomerulonephritis (GN) or lupus erythematosus, to our knowledge, neuroimmune interactions have never been studied in this organ. Renal innervation consists of sympathetic and primary sensory nerve fibers. Renal inflammation may result from primary sensory neurons that evoke neurogenic inflammation or from afferent transmission that probably includes brain-derived neurotrophic factor signaling, or overactivate sympathetic nerves. As mentioned, the sympathetic neurotransmitter norepinephrine exerts pro- and anti-inflammatory properties. In hypertension, excessive sympathetic innervation may produce structural lesions in the kidney. Furthermore, it has been shown that subdepressor doses of sympatholytic agents reduce vascular and glomerular injury and lower albuminuria in rats subjected to subtotal surgical nephrectomy. Despite reports demonstrating a significant role of sympathetic innervation on structural renal damage, its role in kidney inflammation has not yet been defined.

In this study, we investigated the effect of renal innervation on the course of transient anti-Thy-1 GN in the rat, a pathology in which renal inflammation develops independent of primary hypertension. Here, we present data demonstrating a marked ameliorative effect of bilateral renal denervation on the extent of GN-associated renal injury.

**RESULTS**

Renal innervation of sham-operated rats was analyzed in cryostat sections stained for tyrosine hydroxylase (TH) and CGRP to identify sympathetic and primary sensory nerve fibers, respectively. Both types of fibers were observed in the same nerve fiber bundles. TH-positive sympathetic (red) and CGRP-positive primary afferent nerve fibers (green) were detected close to a small renal artery, tubuli (stained light green because of background fluorescence), and glomeruli. TH-positive nerve fibers (red) are more abundant than green CGRP positive neurons (adventitia of an A. arcuata). Two days after unilateral renal denervation, TH- (red) and CGRP-positive (green) paravascular nerve fibers had disappeared from the denervated organ (C) but were still detectable in the nondenervated kidney (arrows in D). Yellow-orange mixed color in A indicates close, partly overlapping vicinity of TH- and CGRP-positive fibers but no co-localization within the same nerve fiber (cf B).

Figure 1. Renal innervation and its depletion by surgical denervation. (A) TH-positive sympathetic (red) and CGRP-positive primary afferent nerve fibers (green) were detected close to a small renal artery, tubuli (stained light green because of background fluorescence), and glomeruli. (B) TH-positive nerve fibers (red) are more abundant than green CGRP positive neurons (adventitia of an A. arcuata). (C and D) Two days after unilateral renal denervation, TH- (red) and CGRP-positive (green) paravascular nerve fibers had disappeared from the denervated organ (C) but were still detectable in the nondenervated kidney (arrows in D). Yellow-orange mixed color in A indicates close, partly overlapping vicinity of TH- and CGRP-positive fibers but no co-localization within the same nerve fiber (cf B). a, artery; g, glomerulus. Bars = 100 \(\mu\)m in A, C, and D and 50 \(\mu\)m in B.

Figure 2. Vicinity of renal nerve fibers to immune cells. ED1-positive macrophages (blue, arrow) are located in close vicinity to both TH- (red) and CGRP-positive (green) nerve fibers. DC (all in blue), positively stained for CD11c, OX62, or OX6 (MHC class II), are closely apposed (arrows) to both TH- (red) and CGRP-positive (green) nerve fibers (purple overlay of TH/OX62 in lower left panel; white triple overlay of OX6, TH, and CGRP in lower right panel; yellow overlay of TH and CGRP nerve fibers as a result of intermingling within the fiber bundles). Bars = 50 \(\mu\)m.
bundles closely apposed to each other along the renal vasculature as well as in the interstitium (Figure 1, A and B). These nerve fibers could not be identified in animals subjected to unilateral denervation 48 h before being killed (Figure 1, C and D). Importantly, sympathetic and primary sensory nerve fibers could be found in close vicinity to ED1-positive macrophages and dendritic cells (DC) positive for CD11c, OX62, or OX6 (Figure 2). While CD11c and OX62 are markers for classic myeloid DC, the CD11c− CD103− (OX62) MHC class II+ (OX6) population represents plasmacytoid DC. Quantitative evaluation revealed that of 100 randomly selected CD11c DC, 28% and 3% had “close” relationships to TH- and CGRP-positive nerve fibers, respectively, whereas 15% and 6% were still found near the respective nerve fibers. The corresponding values for OX62+ cells were 24%/3% and 10%/2% and for ED1+ macrophages were 13%/2% and 8.1%/5.6%, respectively. Of 123 OX6-positive MHC class II expressing cells, 14.6%/9.8% and 8.1%/5.6% displayed “close” and “neighboring” relationships to TH- or CGRP-positive nerve fibers, respectively. Thus, roughly one fourth of DC were found in close apposition to sympathetic nerves, whereas contacts to sensory fibers were significantly less common, probably reflecting the lower incidence of this nerve fiber population in the rat kidney.

For studying the influence of renal denervation on kidney injury in the anti-Thy-1 model of mesangioproliferative GN, bilateral surgical denervation (DNX) and sham operations were carried out 2 d before intravenous injection of the anti-Thy-1.1 antibody (Ab). As shown in Figure 3, albuminuria was significantly reduced 72 h and 6 d after nephritis induction. In both groups, BP was <120 mmHg (mean arterial BP) at the end of the experiments (sham operated + anti-Thy-1.1 versus DNX + anti-Thy-1.1 [107.19 ± 2.03 versus 97.22 ± 4.31 mmHg; P > 0.05]). Accordingly, plasma concentrations of angiotensin II (AngII) were not significantly different between both groups (sham operated + anti-Thy-1.1 versus DNX +
anti–Thy-1.1 [1.25 ± 0.16 versus 3.14 ± 1.36 pg/0.05 ml; P > 0.05]). Renal inflammation was analyzed by determination of macrophage infiltration into glomeruli and interstitium at the end of the experiment (i.e., 6 d after anti–Thy-1.1 mAb injection). By quantification of ED1-positive cells, we observed that macrophage accumulation was significantly reduced in the interstitium of anti–Thy-1.1–treated denervated rats compared with sham-operated controls (Figure 4A), whereas denervation itself did not affect interstitial macrophage counts (sham operated + saline versus DNX + saline [5.45 ± 0.71 versus 7.62 ± 5.47; P > 0.05]). Moreover, glomerular ED1-positive counts were not affected (data not shown); however, expression of the proinflammatory cytokine TNF-α was significantly inhibited in kidneys of anti–Thy-1.1–denervated rats compared with sham-operated controls (Figure 4B). FACS analysis of renal CD11c+ DC in anti–Thy-1.1 rats with or without denervation revealed that their frequency paradoxically increased in denervated rats (Figure 4C). Indeed, comparison of saline controls with anti–Thy-1.1 rats showed that inflammatory kidney disease was associated with a decrease of renal CD11c+ DC frequency, which was partially restored in denervated anti–Thy-1.1 rats (Figure 4C). The frequency of CD11c– MHC class II+ cells, which likely represent the population of T cells that contribute to the pathophysiology of anti–Thy-1 nephritis, increased in anti–Thy-1.1 kidneys compared with those from saline-treated rats and returned to normal in denervated anti–Thy-1.1 rats (sham operated + saline versus sham operated + anti–Thy-1.1 [45.06 ± 0.72 versus 55.92 ± 2.70; P < 0.05]; sham-operated + anti Thy-1.1 versus DNX + anti Thy-1.1 [55.92 ± 2.70 versus 38.58 ± 1.72, P < 0.005]).

Morphologic inspection of mesangiolysis and quantification of microaneurysms (Figure 5A) as well as immunohistochemical analysis and quantification of mesangioproliferation by proliferating cell nuclear antigen (PCNA) staining (Figure 5B) revealed significant evidence of a protective effect of renal denervation on structural damage. A protective effect could also be demonstrated by immunohistochemical analysis and quantification of collagen IV deposition (Figure 6, A and B) and renal expression of TGF-β (Figure 6C), indicating that denervation also prevented renal sclerosis. Taken together, our results demonstrate that renal innervation, consisting of TH-positive sympathetic and CGRP-positive primary sensory nerve fibers, aggravates inflammatory and structural lesions in mesangioproliferative GN.

**DISCUSSION**

The results in our study are the first to demonstrate that renal innervation is critical for the development of experimental nephritis. TH-positive sympathetic nerve fibers and CGRP immunoreactive primary sensory neurons have been previously demonstrated in the renal pelvis and cortex. In accordance with previous findings, we detected CGRP immunoreactivity around arteries, among tubules in the interstitium, and close to glomeruli. TH- and CGRP-positive nerve fibers were found closely apposed to each other and for the first time were shown to lie in very close proximity to inflammatory and antigen-presenting cells, such as ED1-positive macrophages and DC, that positively stain for the classical markers CD11c and OX62 as well as for OX6. It seems likely that among the OX6+ cell population that represents MHC class II expressing cells, a special subset of immature DC (i.e., plasmacytoid DC) is present.
These MHC class II\(^+\) CD11b/c\(^-\) OX62\(^-\) DC, known to produce large amounts of type I IFN upon viral infection, match the human phenotype of plasmacytoid DC.\(^{20}\) Whereas numerous DC have been detected in the tubulointerstitium of mice,\(^{23,24}\) they seem less abundant in rat tubulointerstitium; however, as the immunocytes co-localize with both sympathetic and primary sensory nerve fibers, it is likely that both neuronal systems are involved in neuroimmune interactions in the kidney.

To study the influence of renal innervation on GN, we chose the conventional model of self-limiting, acute normotensive anti-Thy-1 GN\(^{25}\) without unilateral nephrectomy. In this model, the renal inflammatory response results from antibody-dependent mesangial cell cytotoxicity (i.e., from activation of lymphocytes and macrophages before structural lesions), rather than from secondary renal inflammation as a result of primary effects of hypertension. Also, under our experimental conditions, BP did not increase above 120 mmHg throughout the 6-d experiment. Hence, the protective effect of renal denervation observed in this study most likely resulted from abrogation of neuroimmune interactions.

In denervated kidneys, we observed a significant reduction of inflammatory parameters such as accumulation of interstitial macrophages and renal expression of TNF-\(\alpha\); however, when we analyzed the frequency of renal CD11c\(^+\) DC, we found that these cells were paradoxically decreased in anti-Thy-1.1 rats compared with healthy controls. This may have resulted from their migration to draining lymph nodes. Indeed, it has been shown that DC disappear from the renal interstitium within 48 h after administration of LPS to mice. The effect was accompanied by an increase of DC in lymph nodes\(^{26}\); however, we failed to detect trafficking of DC to renal lymph nodes 6 d after anti-Thy-1.1 administration (data not shown). An alternative explanation may be that DC lose their markers after activation, as has been described for other receptors and co-stimulatory molecules on immune cells.

In addition to reduced accumulation of intestinal macrophages and cortical expression of TNF-\(\alpha\), fibrosis was reduced as measured by TGF-\(\beta\) mRNA expression and glomerular collagen IV deposition in denervated anti-Thy-1.1 rats. Moreover, kidney injury as determined by albuminuria, mesangiolysis, and appearance of microaneurysms was significantly reduced. Hence, renal innervation significantly affects disease progression in experimental GN (i.e., the clinical course from inflammation via fibrosis up to structural alterations).

Interestingly, at the time point of the investigation (6 d after anti-Thy-1.1 Ab challenge), macrophage accumulation was significantly reduced in the interstitium but not in glomeruli of denervated rats compared with sham-operated controls. This finding has two important implications. First, it points to the fact that anti-Thy-1.1 nephritis not only is a model of glomerular inflammation but also exhibits an additional strong interstitial inflammatory effect. In this regard, a similar observation was made in nephrotoxic GN mice, where inflammatory cells accumulated within the tubulointerstitium and adjacent to but not within inflamed glomeruli.\(^{27}\) Second, the interstitial portion of the inflammatory response is considerably modulated by the renal autonomic innervation. In the glomeruli, the di-
rect inflammatory effect of the antibody probably overcomes any modulatory effect of the innervation on inflammatory responses. This does not mean, of course, that renal innervation is without any effect on changes within the glomeruli during the anti-Thy-1.1 nephritis, because we observed a significant reduction of glomerular collagen IV deposition and TGF-β expression in denervated rats with anti-Thy-1.1 nephritis. Hence the sclerotic alterations in the glomeruli emerging from massive inflammation upon anti-Thy-1.1 antibody injection are modulated by the renal innervation.

In the kidney, neuroimmune interactions might result from local production of the proinflammatory neuropeptide SP or indirectly via increased cytokine-stimulated sympathetic nerve activity,15 activation of β2 receptors on juxtaglomerular cells by catecholamines, and production of AngII. AngII is widely known as a hypertensive and proinflammatory mediator28; however, we failed to detect significant differences in plasma AngII concentrations between denervated and sham-operated anti-Thy-1.1 rats. This is in accordance with the absence of BP differences; however, these findings do not rule out the possibility that AngII locally acts as one proinflammatory mediator of anti-Thy-1 GN.

Taken together, our results argue for a significant role of the renal innervation for kidney inflammation. This study provides evidence that neurogenic inflammation occurs in the kidney. Therefore, either SP or CGRP receptor antagonists, antagonists blocking ion channels or receptors critical for sensory functions such as the transient receptor potential vanilloid type 1 protein (TRPV1), P2X, bradykinin, protease activated receptors,29 or voltage-gated Na channels30 given alone or in combination might be future options for treatment of inflammatory disease in the kidney.

CONCISE METHODS

Rat Model of Anti-Thy-1.1 Nephritis
Sprague-Dawley rats (Charles River Wiga, Sulzfeld, Germany) were housed in the animal facilities of the Department of Nephrology and Hypertension, University of Erlangen-Nuremberg (Erlangen, Germany). Housing was maintained at 22 ± 2°C, exposed to a 12-h dark/light cycle. The rats were allowed unlimited access to chow (#1320; Altromin, Lage, Germany) and tap water. All procedures performed on rats were performed in accordance with guidelines of the American Physiologic Society and were approved by the local government authorities (Regierung von Mittelfranken).

Two days before induction of nephritis, randomly selected rats were anesthetized with methohexital sodium (Brevimytal; Lilly, Bad Homburg, Germany) intraperitoneally. One group of rats was bilaterally denervated, the others underwent sham operations. For immunohistochemical studies, rats were unilaterally denervated. Bilateral or unilateral flank incisions were made, and renal denervation was performed by surgically stripping the renal arteries and veins of adventitia, cutting all visible renal nerve bundles under a dissection mi-

croscopy (×25), and coating the vessels with a solution of 10% phenol in 95% ethanol as described previously.31 The bilateral renal denervation procedure prevents the renal vasoconstrictor response to supra-lumbar sympathetic nerve stimulation and prevents the antinatriuretic response to environmental stress. It can be demonstrated that this procedure reduces renal catecholamine immunofluorescence to nondetectable levels and reduces renal tissue norepinephrine concentration to <5% of control.32 By the same procedure, also sensory fibers that course in the same nerve bundles intermingled with sympathetic axons are removed.

Anti-Thy-1.1 nephritis was induced by a single intravenous injection of 1 mg/kg monoclonal anti-Thy-1.1 antibody (ER4; Antibody Solutions, Palo Alto, CA). Age-matched controls were given vehicle.

After 6 d, the rats were kept in metabolic cages for determination of 24-h urinary albumin excretion (enzyme immunoassay kit; Cell-Trend, Luckenwalde, Germany). Rats were instrumented with a femoral artery catheter for subsequent measure of arterial BP via transducers (Grass Instruments, Quincy, Massachusetts) connected to a polygraph (Hellige, Freiburg, Germany) 4 h after termination of anesthesia. After rats were killed, the kidneys were removed and decapsulated and the renal tissue was frozen or fixed for histology and immunohistochemistry.

Kidney Histology
For evaluation of histology, renal tissue was fixed overnight in methyl-Carnoy solution (60% methanol, 30% chloroform, and 10% glacial acetic acid) and dehydrated by bathing in increasing concentrations of methanol, followed by 100% isopropanol, and paraffin embedded. Four-micrometer sections were stained with periodic acid-Schiff. For assessment of the extent of the formation of microaneurysms, capillary widening was scored (see statistical analysis).

Immunohistochemistry
Three-micrometer sections of methyl-Carnoy–fixed paraffin-embedded tissue were cut with a Leitz SM 2000 R microtome (Leica Instruments, Nussloch, Germany). After deparaffinization, endogenous peroxidase activity was blocked with 3% H2O2 in methanol for 20 min at room temperature. A mouse mAb that detects proliferating cells (PCNA; Santa Cruz Biotecnologies, Heidelberg, Germany) was used at a dilution of 1:50. The mouse mAb against the macrophage marker ED1 (Serotec, Biozol, Eching, Germany) was used at a dilution of 1:250. A goat polyclonal antibody to collagen IV (Southern Biologicals, Santa Cruz, CA) was used at a dilution of 1:2000. Immunostaining was carried out with a 0.1% diaminobenzidine tetrahydrochloride/0.02% H2O2 detection system (Vector Laboratories, Burlingame, CA). Each slide was counterstained with hematoxylin.

Sympathetic and sensory nerve fiber detection was performed using immunocytochemistry for tyrosine hydroxylase (sheep anti-tyrosine hydroxylase; Novus Biologicals, Littleton, CO; 1:2000) and CGRP (rabbit anti-CGRP; Peninsula, San Carlos, CA; 1:1000), respectively. Relationships of sympathetic and sensory nerve fibers to macrophages and DC were revealed by combining antibodies against these neuronal markers with either ED1 (Serotec, Biozol, Eching, Germany) for macrophages or OX6 (unspecific mouse mAb for MHC class II–expressing cells; Seralab, Balney, United Kingdom; 1:200),
OX62 (Labgen, Frankfurt, Germany; 1:100), and CD11c (Serotec; 1:100) mouse mAb for more specific labeling of DC in triple immunofluorescence. Briefly, 15-μm cryostat sections from formaldehyde perfusion–fixed rat kidneys were incubated with primary antibodies dissolved in TBS containing 1% BSA and 0.5% Triton X100 overnight at room temperature followed by incubation with appropriate fluorochrome-tagged secondary antibodies (donkey anti-rabbit Alexa 488 or 555 and donkey anti-mouse Alexa 488 [both from Molecular Probes; 1:1000] and donkey anti-sheep Cy5 [Dianova, Hamburg, Germany; 1:100]) dissolved in TBS for 1 h at room temperature. Sections were examined with a Biorad MRC 1000 confocal system (Biorad, Hercules, CA) attached to a Nikon Diaphot 300 inverted microscope (Nikon, Düsseldorf, Germany). The blue (488 nm), yellow (568 nm), and far red (647 nm) lines of a krypton-argon laser were used for excitation of Alexa 488, Alexa 555, and Cy5, respectively. The blue 488-nm laser line was also used for eliciting green autofluorescence of various tissue elements in the kidney as a sort of counterstain. Merged two- and three-channel confocal images were adjusted for contrast and brightness using Adobe Photoshop (Adobe, München, Germany).

Quantification of Nerve–Immune Cell Relationships
Randomly selected cryosections were screened in a meander-like fashion for immune cells as labeled with the above mentioned markers. One-hundred cells for each ED1, OX62 and CD11c, and 123 cells for OX6 were evaluated. Whenever immune cells were detected in the vicinity of TH or CGRP positive nerve fibers, a single confocal optical section was scanned. Spatial relationships were determined as “close” (direct apposition or overlap of nerve fibers and immune cells resulting in mixed color in the merge) or “neighboring” (distance between nerve fibers and immune cells from 1 to 10 μm).

Isolation of Lymphocytes from Kidneys and Flow-Cytometric Analysis
After rats were killed, they were perfused with saline and the kidneys were collected. Lymphocytes were isolated from kidneys according to a previously published protocol with modifications. Briefly, kidneys were decapsulated and passed through 100-μm nylon mesh with RPMI 1640. The cells were hydrated with medium supplemented with 5% newborn calf serum. The cell suspension was centrifuged at 300 RPMI 1640. The cells were hydrated with medium supplemented with 37% Percoll solution (Cytomix, Munich, Germany) and passed through 100-μm nylon mesh with RPMI 1640. The cells were hydrated with medium supplemented with 5% newborn calf serum. The cell suspension was centrifuged at 300 × g for 10 min, and the pellet was resuspended with 37% Percoll solution (Amersham-Biosciences, Freiburg, Germany) containing 100 U/ml heparin and centrifuged at 800 × g for 20 min. Leukocytes (5 × 10⁷) were stained using a standard protocol including preblocking of Fc receptors. For detection of DC, a FITC-labeled anti-rat CD11c antibody (Serotec, Düsseldorf, Germany) and a PE-labeled anti-rat MHC II antibody (Serotec) were used. Data were recorded and analyzed using a FACScan Flow Cytometer (BD Biosciences, San Jose, CA) and Cellquest Software (BD Biosciences, San Jose, CA).

Measurement of Plasma AngII
Plasma AngII was measured by direct RIA with an antibody 100% cross-reactive with AngIII and IV as described previously. Real-Time Reverse Transcriptase–PCR Detection of mRNA
Renal cortical tissue extraction and real-time reverse transcriptase–PCR were carried out as described previously. Primer sequences for TGF-β1 were described previously. The relative amount of the specific mRNA was normalized to the housekeeping gene β-actin. Primer sequences were as follows: rat β-actin 5′-GCC TTC CCT CCT GGG TAT G and 3′-TCA GGA GGA GCA ATG ATC TTG and rat TNF-α 5′-GTC GTA GCA AAC CAC CAA G and 3′-GAG CAA TGA CTC CAA AGT AG.

Statistical Analysis
Intraglomerular PCNA- or ED1-positive cells were counted in all glomeruli of a given kidney section (120 to 300 glomeruli, no selection) and expressed as cells per glomerular section. Areas of the glomeruli evaluated were not different between the denervated and nondenervated rats treated with Anti–Thy-1.1. Interstitial PCNA- or ED1-positive cells were counted in 30 medium-power (magnification ×250) cortical views per section and expressed as cells per mm². Glomerular collagen IV staining was measured by Metaview (Visithron Systems, Puchheim, Germany) in every third glomerulus per cross-section, and the stained area was expressed as percentage of the total area of the glomerular tuft. For assessment of the extent of the formation of microaneurysms, capillary widening was scored using a scale from 0 to 4: 0, no capillary widening in the glomerular tuft; 1, capillary widening involving up to 25% of the glomerular tuft; 2, capillary widening 25 to 50%; 3, capillary widening 50 to 75%; and 4, capillary widening involving >75% of the glomerular tuft.

Two-way ANOVA, followed by the post hoc least significant difference test, was used to compare groups. P < 0.05 was considered significant. The procedures were carried out using the SPSS 13.0 software (SPSS, Chicago, IL). Values are displayed as means ± SEM.

ACKNOWLEDGMENTS
This work was supported by a grant of the Deutsche Forschungsgemeinschaft to R.V. and G.T. (SFB 423 B12).

The expert technical assistance of Sonja Heinlein, Karin Löschner, and Hedwig Symovski is gratefully acknowledged.

DISCLOSURES
None.

REFERENCES


