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Original Paper

Renal expression of endothelial and inducible nitric oxide synthase, and formation of peroxynitrite-modified proteins and reactive oxygen species in Wegener's granulomatosis

Peter Heeringa*, Marc Bijl, Alie de Jager-Krikken, Andre Zandvoort, Gerard Dijkstra, Han Moshage, Jan W. Cohen Tervaert, Anton T. M. G. Tiebosch, Cees G. M. Kallenberg and Harry van Goor
Department of Clinical Immunology, Pathology and Division of Gastroenterology and Hepatology, University Hospital Groningen, The Netherlands

*Correspondence to:
P. Heeringa, Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, Brinkhous-Bullitt Building, Room #818, CB# 7525, Chapel Hill, NC 27599-7525, USA.
E-mail:
Peter_Heeringa@med.unc.edu

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Abstract

To investigate the role of nitric oxide (NO) in glomerular inflammation, the expression of endothelial NO synthase (eNOS) and inducible NOS (iNOS) was studied in conjunction with inflammatory cell influx, H_2O_2 production, and the formation of nitrotyrosines in renal biopsies from patients with Wegener's granulomatosis (WG). Renal cryostat sections from patients with WG ($n=15$) were stained by immunohistochemistry for eNOS, iNOS, endothelial cells (CD31), nitrotyrosines, polymorphonuclear cells (PMNs, CD15), and monocytes/macrophages (CD14, CD68). Production of H_2O_2 was identified by enzyme cytochemistry using diaminobenzidine. In control tissues, strong staining for eNOS was found in glomerular and interstitial tubular capillaries and cortical vessels. A significant reduction in eNOS expression was found in WG biopsies, which was associated with a reduction in CD31 expression. Expression of iNOS was found in infiltrating inflammatory cells, mainly located in the interstitium. H_2O_2-producing cells were detected in glomeruli and were abundantly present in the interstitium. Nitrotyrosine-positive cells, however, were almost exclusively found in the interstitium. It is concluded that renal inflammation in WG is associated with the induction of iNOS in inflammatory cells and the formation of nitrotyrosines. Expression of eNOS in glomerular capillaries is lost, most likely due to endothelial cell damage. These results suggest that decreased $NO^{\cdot}$ production by endothelial cells, in conjunction with increased $NO^{\cdot}$ production by iNOS-positive inflammatory cells, is involved in renal tissue injury in WG. Copyright © 2000 John Wiley & Sons, Ltd.

Keywords: **Wegener's granulomatosis; nitric oxide synthases; nitrotyrosine; oxygen radicals**

Introduction

The production of nitric oxide (NO) radicals has been recognized as an important mediator system in various physiological and pathophysiological processes. NO is produced from the terminal guanidine nitrogen of L-arginine by nitric oxide synthases (NOSs), of which three isoforms have been identified encoded by distinct genes: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). In the kidney, nNOS is expressed in the macula densa, where it may be involved in the regulation of the vascular tone of the afferent and efferent arterioles [2]. Expression of eNOS is predominantly found in endothelial cells and is important in the regulation of vascular tone [2]. NO generated by eNOS has also been shown to inhibit platelet aggregation and platelet and leukocyte adhesion to the endothelium [3]. Both nNOS and eNOS are calcium- and calmodulin-dependent and are constitutively expressed, generating small amounts of NO (pmol) [3]. In contrast, iNOS is a calcium- and calmodulin-independent enzyme expressed only after exposure to inflammatory stimuli in a variety of cell types including intrinsic renal cells [4,5]. Once induced, iNOS is capable of producing large amounts of NO (μmol) for prolonged periods of time.

In experimental models of glomerulonephritis, increased NO production has been demonstrated, most likely derived from infiltrating inflammatory cells expressing iNOS [5,6]. High concentrations of NO generated by iNOS may have cytotoxic effects. NO may also react with superoxide anions, yielding the highly reactive compound peroxynitrite. Peroxynitrite may initiate lipid peroxidation and induces nitration of tyrosine residues, leading to loss of protein structure and function [7,8].

Wegener's granulomatosis (WG) is a systemic inflammatory disease characterized by necrotizing granulomatous inflammation and necrotizing small vessel vasculitis; it is associated with the presence of anti-neutrophil cytoplasmic antibodies (ANCAs) directed against either proteinase 3 (Pr3) or myeloperoxidase (MPO) [9]. A rapidly progressive form of glomerular disease occurs in most patients, characterized by fibrinoid necrosis of the glomerular capillaries, the formation of glomerular crescents, and marked

glomerular and interstitial infiltration of neutrophils and mononuclear leukocytes, sometimes accompanied by granuloma formation [10,11].

In a rat model of anti-MPO-associated crescentic glomerulonephritis (CGN), we recently investigated the temporal expression of NO synthases in conjunction with platelet aggregation, inflammatory cell influx, and the generation of reactive oxygen species (ROS) and nitrotyrosine formation. These studies showed that eNOS expression was markedly decreased and was associated with extensive platelet aggregation. A marked transient induction of iNOS in PMNs and macrophages was observed, coinciding with the generation of ROS and the formation of nitrotyrosines [12]. These studies suggested that NO generated by eNOS is protective, whereas NO generated through iNOS is involved in tissue injury in this model of anti-MPO-associated CGN. To extend our investigations to the role of NO radicals in glomerular inflammation, we have now studied the expression of eNOS, iNOS, and H_2O_2-producing cells in conjunction with the infiltration of inflammatory cells and the formation of nitrotyrosines in renal biopsies from patients with WG.

Materials and methods

Biopsies

Fifteen renal biopsies were studied from patients with newly diagnosed WG, as established by the presence of clinical symptoms and histopathological findings. All patients fulfilled the American College of Rheumatology classification criteria for WG [13] and tested positive for anti-neutrophil cytoplasmic antibodies which were directed to Pr3 ($n=9$) or MPO ($n=6$) as tested by indirect immunofluorescence on ethanol-fixed neutrophils and capture ELISA [14]. All biopsies were taken before therapy had started. Clinical data of the patients are shown in Table 1. Tissue specimens ($n=6$) from uninvolved areas of adenocarcinomatous kidneys were used as normal controls.

Frozen sections

For immunohistochemistry, renal tissue was embedded in Tissue-Tek (Sakura Finetek, Europe, Zoeterwoude, The Netherlands), snap-frozen in liquid nitrogen, and stored at -80°C. Consecutive cryostat sections were cut at 4 μm.

Detection of iNOS and eNOS

Cryosections were fixed in acetone for 10 min at room temperature. For the detection of iNOS and eNOS, mouse monoclonal antibodies were used (both from Transduction Laboratories, Lexington, KY, USA; catalogue No. N39120 and N30020, respectively). Endogenous peroxidase was blocked by treatment with 0.075% H_2O_2 in phosphate-buffered saline (PBS) for 20 min. Binding was detected with a sensitive three-step immunoperoxidase technique using sequential incubations of peroxidase (PO)-labelled rabbit anti-mouse IgG, PO-labelled goat anti-rabbit IgG, and PO-labelled rabbit anti-goat IgG secondary antibodies (all from Dakopatts, Glostrup, Denmark). All antibody dilutions were made in PBS supplemented with 1% (v/v) BSA and 1% (v/v) normal human serum. Aminoethylcarbazole (AEC) and H_2O_2 were used as the substrate and sections were counterstained with haematoxylin.

As a positive control for iNOS staining, mouse macrophages (RAW 167) stimulated with mouse interferon γ (IFNγ; 200 U) and LPS (1 μg) for 24 h and human monocytes isolated from whole blood according to the method described by Freundlich and Avdalovic [15] and stimulated with human IFNγ (500 U) for 3 days were stained for iNOS as described above. Lysates of the stimulated cells were run on a 10% SDS-PAGE gel under non-reducing conditions

Table 1. Patient characteristics

Patient No.	Age (years)	Sex	ANCA specificity	ANCA titre	Creatinine (μmol/l)	CRP (mg/l)	WBC ($\times 10^9$/l)	Proteinuria (g/24 h)
1	42	M	Pr3	320	278	44	40.4	3.1
2	62	M	Pr3	640	351	104	1.9	0.5
3	70	M	Pr3	>640	591	108	18.2	3
4	63	M	Pr3	>640	190	115	10.2	2.6
5	54	M	Pr3	640	524	177	9.9	1.7
6	58	M	Pr3	640	252	24	6.4	1.8
7	71	M	Pr3	320	243	191	14.1	4.1
8	67	M	Pr3	160	244	28	5.8	1.4
9	70	F	Pr3	>640	534	179	11.8	2.8
10	69	F	MPO	320	316	5	4.6	1
11	73	M	MPO	640	361	154	8	0.9
12	67	M	MPO	640	552	27	9	1.7
13	21	F	MPO	>640	120	113	8.9	4.5
14	37	F	MPO	>640	866	62	6.1	6.5
15	53	M	MPO	640	723	42	9.9	1.8

F = female; M = male; Pr3 = proteinase 3; MPO = myeloperoxidase; ANCA = anti-neutrophil cytoplasmic autoantibody; CRP = C-reactive protein; WBC = white blood cells.

and transferred to nitrocellulose. After blocking in 4% non-fat skim milk/PBS/Tween-20 (0.1%, v/v)), blots were probed with the monoclonal anti-iNOS antibody in PBS/Tween-20. Bound antibody was detected with PO-labelled rabbit-anti mouse IgG (Dakopatts) followed by PO-labelled swine anti-rabbit IgG (Dakopatts). The reaction was visualized with the enhanced chemoluminescence (ECL) western blotting system (Amersham International, Buckinghamshire, UK)

The glomerular expression of eNOS was evaluated for each glomerulus using a semiquantitative scoring system; 0, absent; 1, minimal; 2, mild; 3, moderate; 4, strong. Values represent mean scores per biopsy. For iNOS, the numbers of positive cells per glomerulus were counted. Interstitial iNOS expression was evaluated using a semiquantitative scoring system: 0, absent; 1, minimal numbers; 2, few; 3, moderate numbers; 4, numerous.

Detection of nitrotyrosine formation

Cryosections were fixed in acetone for 10 min at room temperature and incubated with an affinity-purified rabbit polyclonal antibody (Upstate Biotechnology, Lake Placid, NY, USA; catalogue No. 06-284) against nitrotyrosine. Peroxidase-conjugated goat anti-rabbit IgG (Dakopatts) was used as a secondary antibody. The total numbers of positive intraglomerular cells were counted in each biopsy and expressed as the mean number of positive cells per glomerular cross-section. Interstitial positive cells were quantified by counting positive cells in five microscopic fields at 20× magnification. Results are expressed as the mean number of positive cells per microscopic field.

Detection of H_2O_2 production

A cytochemical technique was used to detect H_2O_2 production [14]. Sections were incubated with diaminobenzidine (DAB) in PBS at 37°C for 30 min. Sections were counterstained with haematoxylin. The total numbers of positive intraglomerular cells were counted and expressed as the mean number of positive cells per glomerular cross-section. Interstitial positive cells were quantified by counting positive cells in five microscopic fields at 20× magnification. Results are expressed as the mean number of positive cells per microscopic field.

Detection of inflammatory cells, endothelial cells, and platelets

Cryosections were fixed in acetone for 10 min at room temperature. To detect infiltrating monocytes/macrophages and polymorphonuclear cells (PMNs), monoclonal antibodies directed against CD68 (KP-1, Dakopatts), CD14 (LeuM3, Beckton Dickinson), and CD15 (Leu M1, Beckton Dickinson) were used. For the detection of endothelial cells and platelets, monoclonal antibodies directed against CD31 and CD41 (Dakopatts) were used. Binding of the antibodies was detected using sequential incubations with PO-labelled rabbit anti-mouse IgG and PO-labelled goat anti-rabbit IgG and colour was developed using AEC and H_2O_2.

The total numbers of intraglomerular CD14- and CD15-positive cells were counted and are expressed as the mean number of positive cells per glomerular cross-section. Interstitial infiltration of CD68-, CD14-, and CD15-positive cells was scored on a semiquantitative scale: 0, absent; 1, minimal numbers; 2, few; 3, moderate numbers; 4, numerous.

Statistical analysis

Values are expressed as mean ± SD. Comparisons between groups were made with the two-tailed Mann–Whitney *U*-test. Correlations within groups were made with Spearman's rank correlation test.

Results

Expression of eNOS

In control tissue, strong staining for eNOS was found in cortical vessels and glomerular capillaries (Figures 1A and 2). In contrast, glomerular eNOS expression in WG biopsies was significantly diminished (Figures 1B, 1C, 1D, and 2). In most cases, the decrease in glomerular eNOS expression coincided with a decrease in glomerular CD31 expression, suggesting that the decreased eNOS expression is due to endothelial cell injury (Figures 3A and 3B). In some glomeruli, however, eNOS expression was decreased, whereas CD31 was still strongly expressed (Figures 3C and 3D). By immunohistochemistry, only minor amounts of platelets could be detected.

Expression of iNOS

iNOS expression was found in mouse macrophages (RAW) cells 24 h after treatment with LPS/IFNγ. In isolated human monocytes, iNOS expression was observed 3 days after treatment with IFNγ, whereas untreated monocytes were negative (Figures 4A and 4B). By western blot, a single band of 130 kD was detected in stimulated mouse macrophages and stimulated human monocytes (Figure 4C). In control tissue, no glomerular iNOS expression could be detected, whereas in the interstitium an occasional cell positive for iNOS was found. In contrast, cells positive for iNOS were clearly detected in the WG biopsies. The majority of these cells was found in the interstitium (Figures 5C and 6), sometimes surrounding glomeruli (Figures 5A and 5B). CD14- and CD68-positive cells were also mainly found in the interstitium (Figure 6). Comparing consecutive sections, an overlap was found between iNOS-positive cells and CD14- or CD68-positive cells, although CD14 and CD68 positivity was always more extensive.

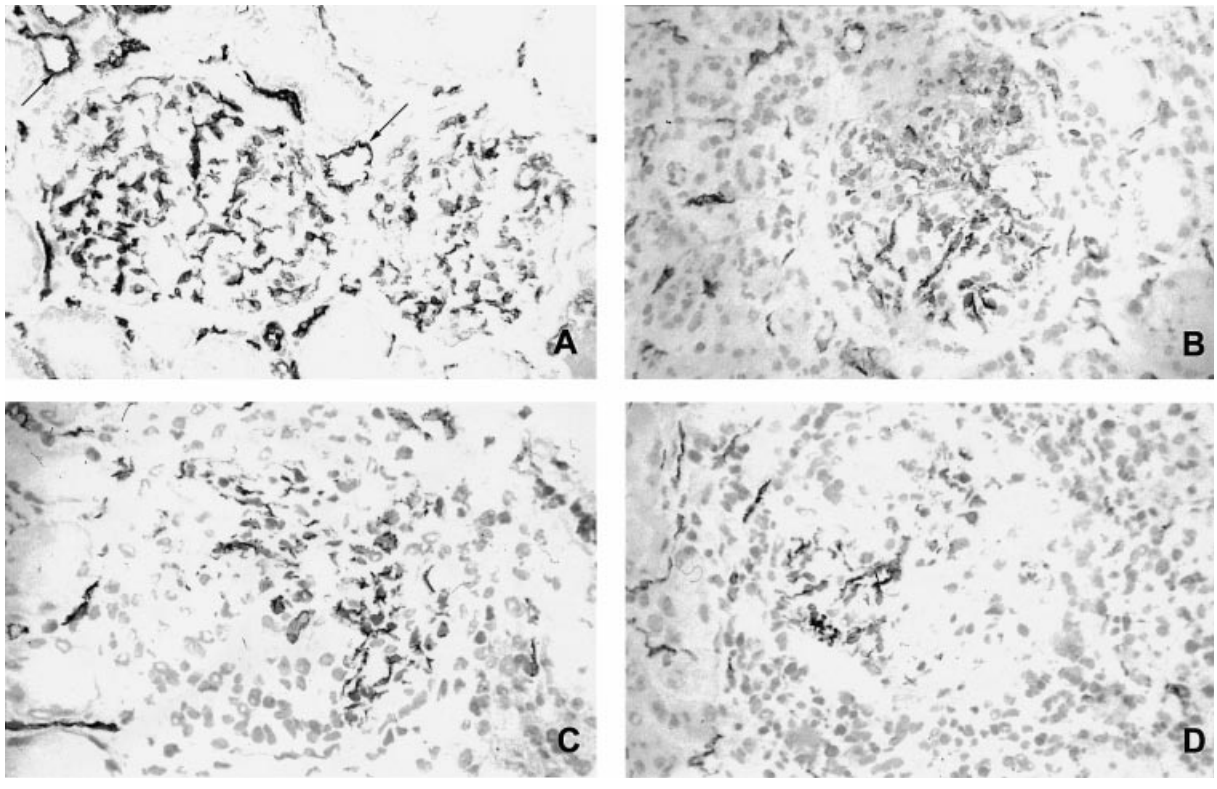

Figure 1. Immunohistochemistry for endothelial nitric oxide synthase (eNOS). (A) Expression and localization of eNOS in control renal tissue. Strong eNOS immunoreactivity is observed in glomerular capillaries and tubular capillaries (arrows). (B, C, D) eNOS immunoreactivity in renal tissue from WG patients, illustrating different degrees of reduction in glomerular eNOS expression. Using the semiquantitative scoring system, these glomeruli would score 3+, 2+, and 1+ in B, C, and, D respectively. (A) ×270; (B–D) ×360

Expression of nitrotyrosine-positive and H_2O_2-positive cells

In WG biopsy specimens, enzyme cytochemistry revealed a significant increase in the number of H_2O_2-producing cells in glomeruli and interstitium compared with control tissue (Figures 7A, 7C, 7E, and 8). The number of glomerular and interstitial H_2O_2-producing cells correlated weakly with serum creatinine levels ($r=0.45$, $p<0.05$ and $r=0.47$, $p<0.05$, respectively). Staining for CD15 in consecutive sections suggested that some of the H_2O_2-producing cells were PMNs. However, not all CD15-positive cells produced H_2O_2 and not all H_2O_2-producing cells were CD15-positive. Expression of nitrotyrosines was almost exclusively found in cells located in the interstitium (Figures 7B, 7D, and 8). Double labelling experiments suggested that all nitrotyrosine-positive cells were also H_2O_2-producing cells, but not all H_2O_2-producing cells were nitrotyrosine-positive.

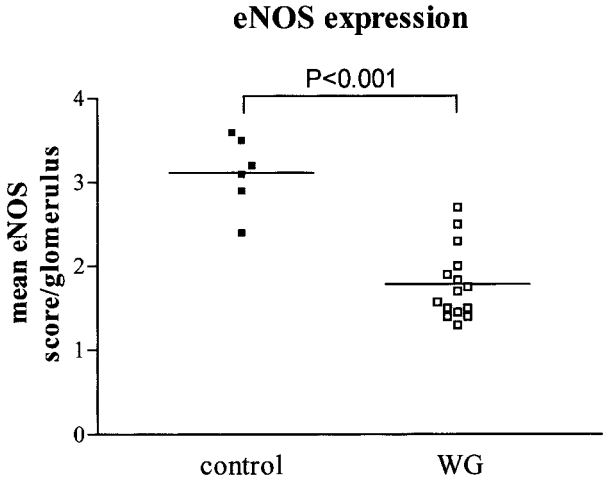

Figure 2. Mean eNOS immunoreactivity score in control and WG renal biopsies. Immunoreactivity for eNOS was scored on a semiquantitative scale: 0, absent; 1, weak; 2, mild; 3, moderate, 4, strong. Values represent the mean score per biopsy

Discussion

In the present study, we investigated the expression of the nitric oxide synthase isoforms eNOS and iNOS in conjunction with the presence of H_2O_2-producing cells, the infiltration of inflammatory cells, and the formation of nitrotyrosines, in renal biopsies from patients with WG. The results show a marked reduction in glomerular expression of eNOS compared with normal human renal tissue. These data confirm and extend the observations by Furusu *et al.*, who reported loss of eNOS immunoreactivity in human glomerulonephritides, which correlated with the severity of glomerular injury [16]. However, it is unclear whether loss of eNOS expression is the cause or the result of

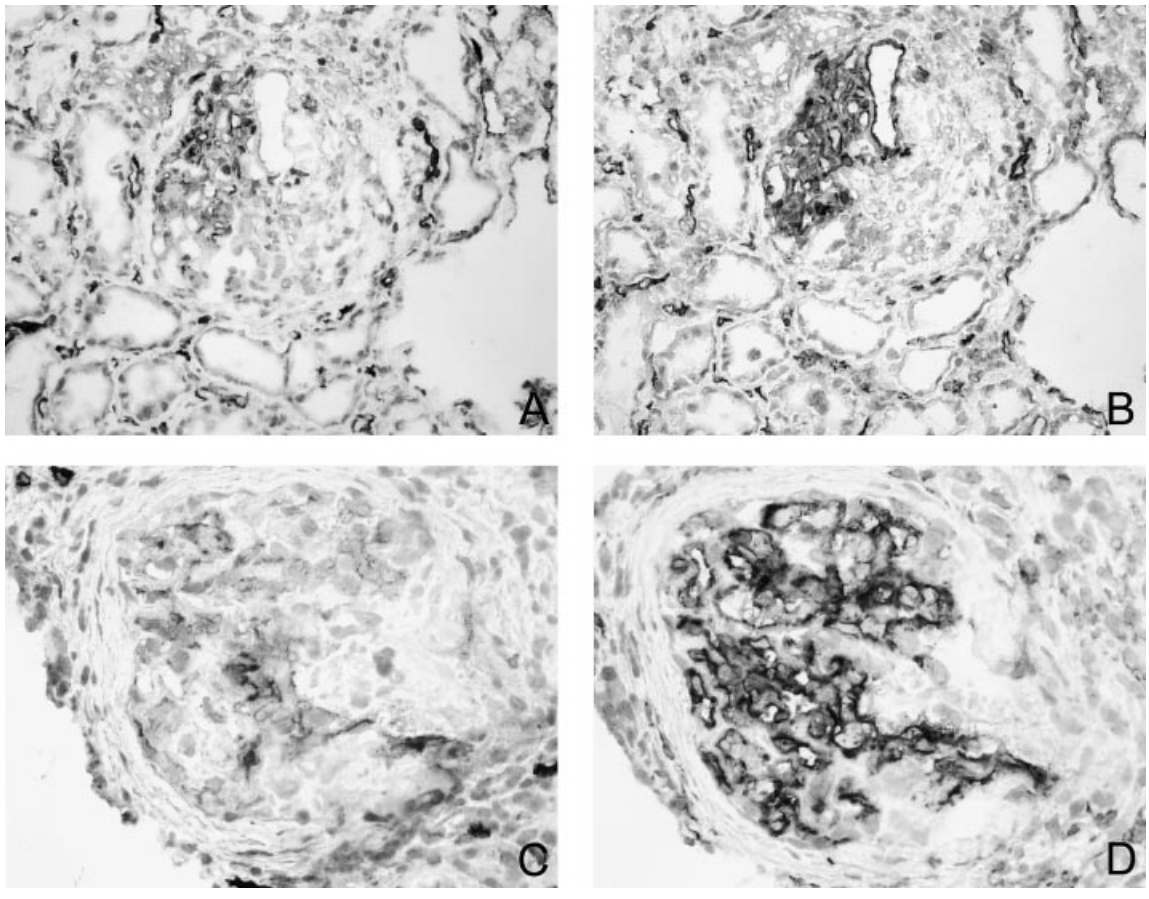

Figure 3. Immunohistochemistry for CD31 and endothelial nitric oxide synthase (eNOS) in consecutive sections. (A, B) Glomerulus showing absence of eNOS immunoreactivity (A) in part of the glomerulus, coinciding with loss of CD31 expression (B). (C, D) Glomerulus showing decreased expression of eNOS (C), whereas CD31 is still strongly expressed (D). (A, B) $\times$180; (C, D) $\times$360

glomerular injury in WG and other glomerulonephritides. In the present study, decreased eNOS expression was found to be associated with decreased expression of CD31, suggesting that loss of eNOS expression is primarily due to endothelial cell injury. These results are in agreement with our previous studies in a rat model of anti-MPO-associated necrotizing glomerulonephritis, in which a marked decrease in glomerular eNOS immunoreactivity was found to be associated with loss of endothelial cell staining [12]. NO radicals generated by constitutively expressed eNOS play an important role in the regulation of the glomerular microcirculation by modulating vascular tone in the afferent arterioles [17,18]. In addition, NO radicals generated by eNOS are essential in maintaining the anti-thrombogenic and anti-inflammatory properties of the endothelium. Consequently, loss of eNOS expression in injured glomeruli may result in disturbances in vascular tone and may decrease the anti-thrombogenic and anti-inflammatory potential of the endothelium. As such, loss of eNOS is detrimental and may exacerbate the progression of glomerular inflammation.

In WG biopsies, a marked induction of iNOS was found in inflammatory cells primarily located in the interstitium and the periglomerular area. Although the exact cellular source for iNOS was not identified, immunostaining with antibodies against CD14 (monocytes) and CD68 (macrophages) in consecutive sections suggested that iNOS was mainly expressed in monocytes and macrophages. These results are in agreement with the data of Kashem *et al*., who in renal biopsies from patients with IgA nephropathy and with non-IgA mesangial proliferative glomerulonephritis also demonstrated iNOS expression in tubulointerstitial areas where infiltration with monocytes/macrophages was abundant [19]. In addition, iNOS expression by monocytes/macrophages has been demonstrated in experimental models of glomerulonephritis [6]. Although the mediators for iNOS expression have not been identified here, it is expected that (pro)inflammatory cytokines such as interleukin-1β (IL-1β), tumour necrosis factor α (TNFα), and IFNγ, or combinations thereof are involved. It is well documented that these cytokines can induce iNOS expression in monocytes/macrophages *in vitro* and their production in active inflammatory lesions as seen in WG is likely [4].

There is considerable controversy regarding the role of iNOS in glomerular inflammation. Narita *et al*. have demonstrated that in the anti-thy-1 model of glomerulonephritis, administration of the NOS inhibitor L-NMMA or restriction of dietary L-arginine prevented

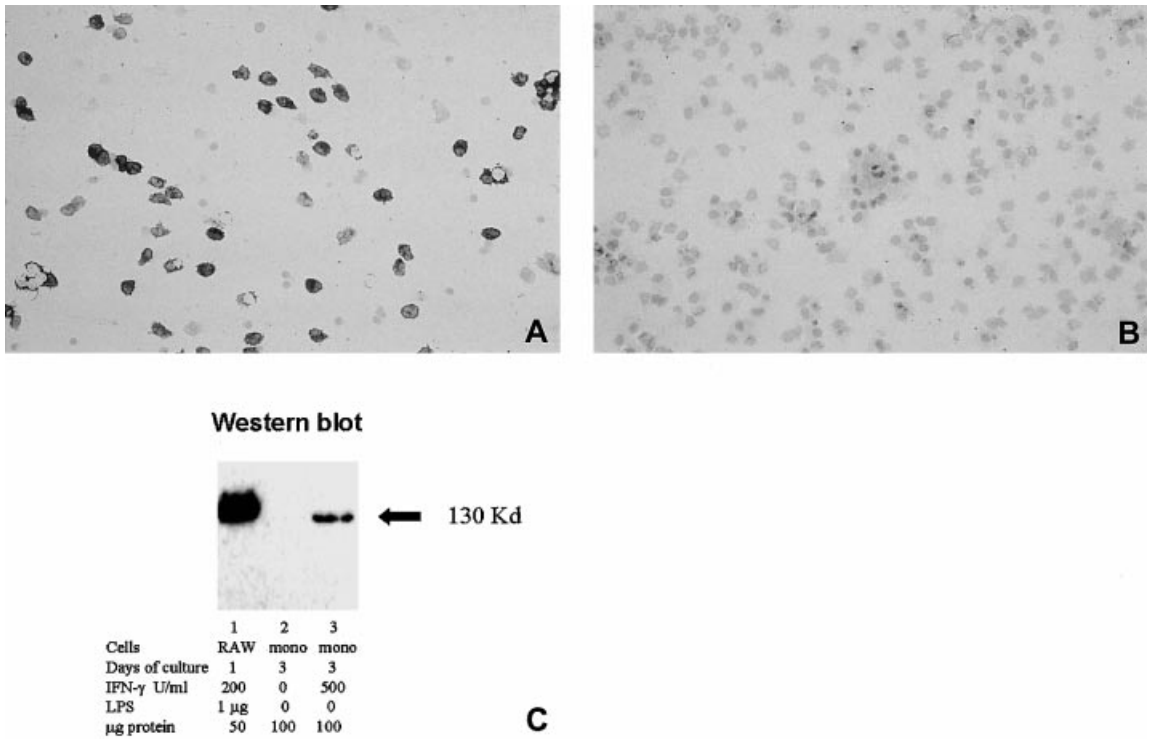

Figure 4. Expression of inducible nitric oxide synthase (iNOS) in isolated human monocytes. (A) Strong expression of iNOS in isolated human monocytes stimulated with IFNγ (500 U) for 3 days. (B) Non-stimulated monocytes cultured for 3 days are negative. (C) Western blot analysis on cell extracts revealed a 130 kD band in RAW cells stimulated with LPS (1 µg) and IFNγ (200 U, lane 1) and isolated monocytes stimulated for 3 days with IFNγ (500 U, lane 3), whereas no band was detected in unstimulated monocytes cultured for 3 days (lane 2). (A, B) $\times 85$

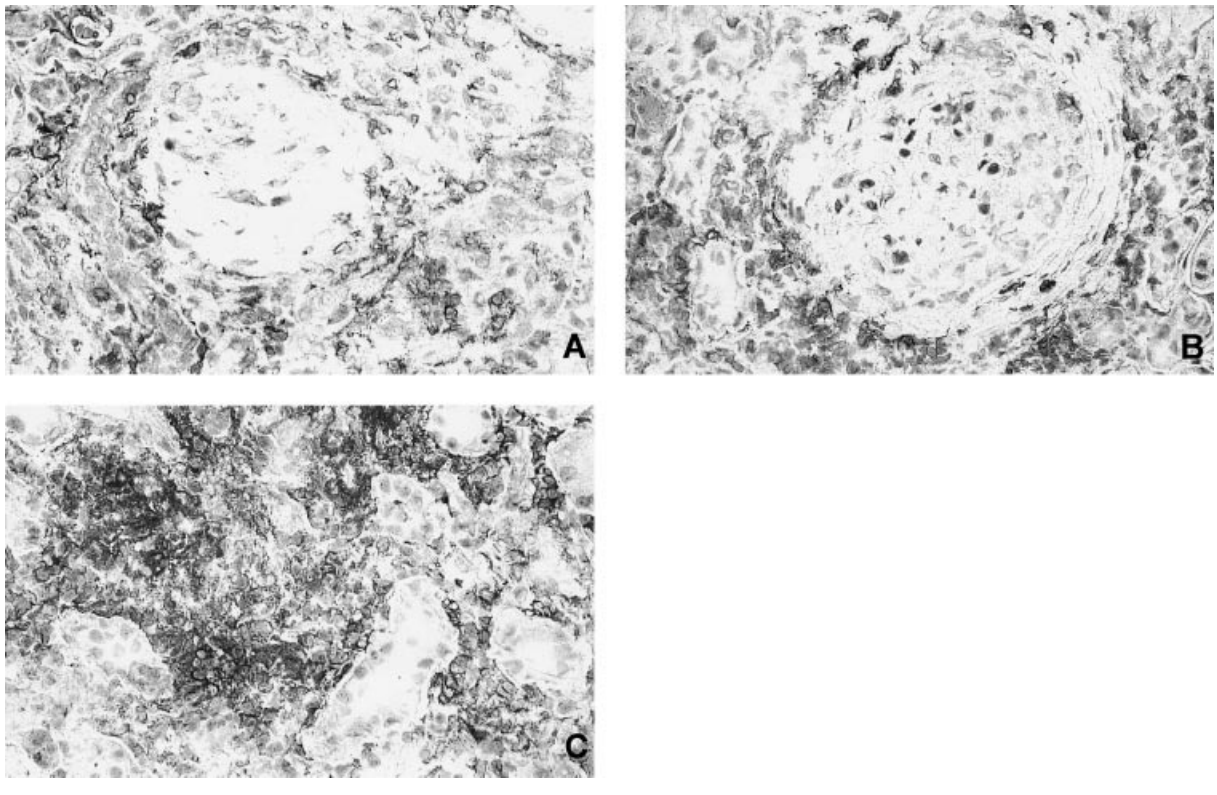

Figure 5. Immunoreactivity for iNOS in renal biopsies from WG patients. (A, B) Periglomerular localization of iNOS-positive cells. (C) Accumulation of iNOS-positive cells in the interstitium. (A–C) $\times 360$

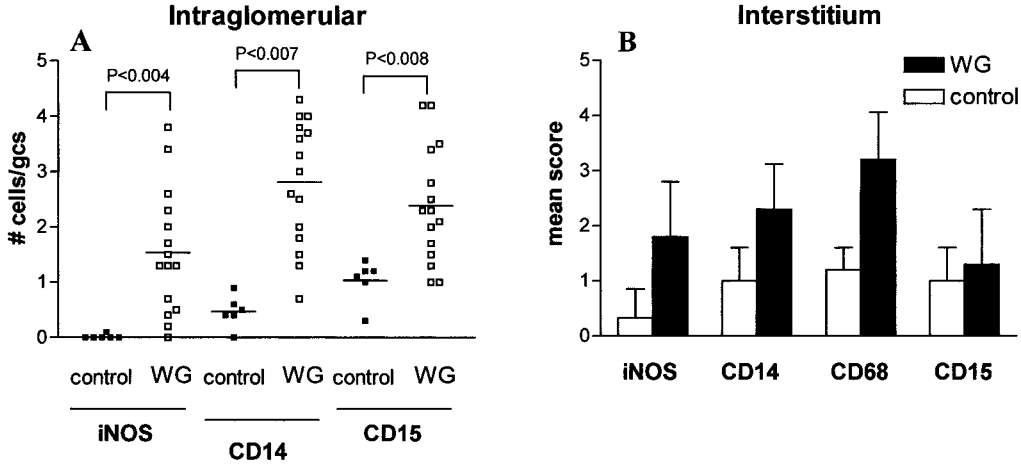

Figure 6. Quantification of intraglomerular (A) and interstitial (B) iNOS, CD14, CD15, and CD68 positive cells. Intraglomerular cells were quantitated by counting the number of positive cells per glomerulus. Values represent the mean number of positive cells

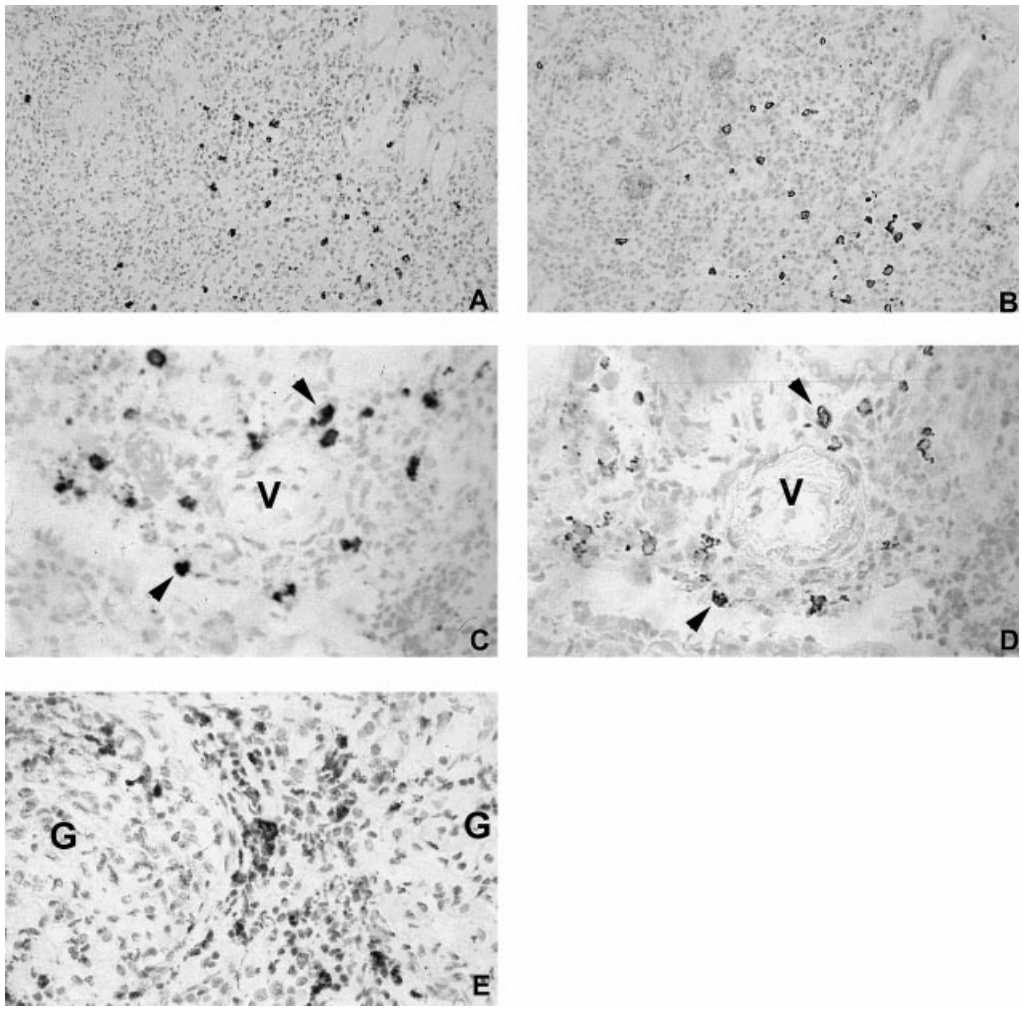

Figure 7. Expression and localization of H_2O_2-producing cells and nitrotyrosine-positive cells. (A) Interstitial localization of H_2O_2-producing cells. (B) Interstitial localization of nitrotyrosine-positive cells. (C, D) Small vessel (V) in the interstitium surrounded by H_2O_2-producing cells (C) and in a consecutive section by nitrotyrosine-positive cells (D). H_2O_2-producing cells positive for nitrotyrosines are indicated by the arrow-heads. (E) Intra- and periglomerular presence of H_2O_2-producing cells. (A, B) $\times 90$; (C–E) $\times 360$

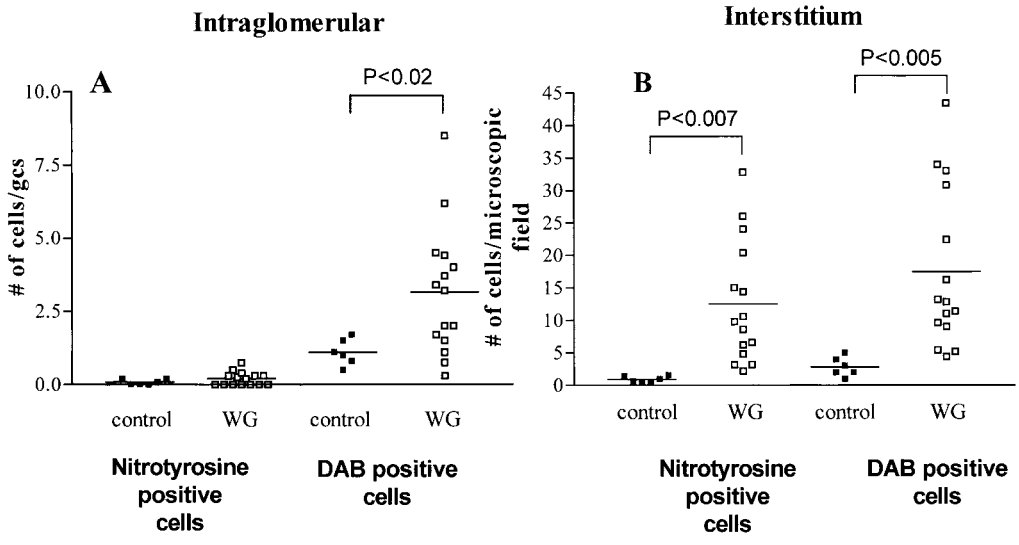

Figure 8. Quantitation of intraglomerular (A) and interstitial (B) H_2O_2-producing cells and nitrotyrosine-positive cells. Intraglomerular cells were quantitated by counting the number of positive cells per glomerulus. Values represent the mean number of positive cells per glomerular cross$_2$-section. Interstitial positive cells were quantified in each biopsy by counting positive cells in five microscopic fields at 20 × magnification. Results are expressed as the mean number of positive cells per microscopic field

mesangial cell lysis [20]. In addition, Bremer *et al*. have shown a beneficial effect of treatment with L-NAME, a non-specific NOS inhibitor, and aminoguanidine, a relatively specific iNOS inhibitor, on glomerular injury rat nephrotoxic nephritis [21]. In contrast, L-arginine depletion leads to increased proteinuria and glomerular thrombosis in rat nephrotoxic nephritis and administration of L-NAME in LPS-treated rats induces generalized glomerular thrombosis [22,23]. Furthermore, Cattell *et al*. showed that the absence of iNOS does not affect the course of accelerated nephrotoxic nephritis in mice [24]. Part of the conflict of data regarding NO inhibition in models of glomerulonephritis probably results from the use of non-selective NOS inhibitors. However, it may also indicate a more complex role for iNOS in glomerular inflammation, which may depend on the cell type(s) expressing iNOS and the interaction of NO radicals with other oxygen radicals.

Increased production of NO radicals may be cytotoxic or cytostatic by binding of NO to iron–sulphur-containing enzymes, particularly those of the mitochondrial electron transport chain [3]. NO may also react with superoxide anions, leading to the formation of peroxynitrite. Peroxynitrite is a potent oxidant capable of initiating lipid peroxidation and inducing nitration of tyrosine residues, leading to loss of protein structure and function [7,8]. Here, immunostaining with a specific antibody for nitrotyrosine residues, as a marker for *in vivo* peroxynitrite formation, revealed intense immunoreactivity in oxygen radical-producing inflammatory cells, which were almost exclusively located in the interstitium. These observations are in agreement with our previous studies in the rat model of anti-MPO-associated glomerulonephritis, in which nitrotyrosine-positive cells showed a similar localization and association with oxygen radical-producing inflammatory cells [12]. Recently, it has been demonstrated that peroxynitrite mediates IL-8 gene expression and IL-8 production in LPS-stimulated peripheral blood leukocytes and is an effective priming agent for neutrophils, leading to enhanced superoxide production and increased intracellular calcium levels [25,26]. The priming effects of peroxynitrite on PMNs were associated with tyrosine nitration of PMN proteins [26]. In addition, peroxynitrite induces apoptosis and modulates heme-oxygenase-1 expression in vascular endothelial cells [27]. Furthermore, peroxynitrite inhibits T-lymphocyte activation and proliferation, which is associated with decreased tyrosine phosphorylation and increased apoptosis [28]. Taken together, these studies suggest that the formation of peroxynitrite at sites of inflammation may play an important modulatory role in inflammatory processes.

In conclusion, our results indicate that loss of NO production by eNOS in conjunction with increased NO production by iNOS-positive inflammatory cells and the formation of nitrotyrosines are involved in the development of renal tissue injury in WG. These observations are compatible with a protective role for eNOS, contrasting with the harmful effects of iNOS during renal inflammation in WG. [1]

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