Clin Exp Immunol 1994; 97:315–320

Expression of the gene for inducible nitric oxide synthase in experimental glomerulonephritis in the rat

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(*Accepted for publication 10 May 1994*)

SUMMARY

Nitrite, a stable product of nitric oxide (NO), is synthesized *in vitro* by glomeruli in experimental glomerulonephritis. We have now studied the expression of the gene for inducible NO synthase (iNOS) in accelerated nephrotoxic nephritis (NTN). The purpose of the study was to confirm *in vivo* induction of iNOS in this model of immune complex disease, and to relate the onset of induction and the level of expression to pathogenic events in the model. Glomeruli from rats with NTN were isolated at 6 h, 24 h and 2, 4 and 7 days and total RNA extracted. RNA (10 μg) was reverse transcribed and polymerase chain reaction (PCR) was performed with primers homologous to rat vascular smooth muscle iNOS and rat β actin. A 222-base PCR product corresponding to iNOS mRNA was present in all experimental animals. iNOS expression was also found in activated macrophages, neutrophils and IL-1-stimulated but not unstimulated mesangial cells. Quantitative competitive PCR was carried out on glomerular samples using a 514-bp mutant of a 735-bp PCR product. iNOS expression was present at low levels in normal glomeruli and was markedly enhanced at 6 h after the induction of glomerulonephritis and peaked at 24 h. Increased iNOS expression persisted to day 7. β actin mRNA levels were similar in all glomerular specimens. This study demonstrates that there is *in vivo* induction of iNOS in immune complex glomerulonephritis, corresponding to the generation of nitrite we have previously reported. iNOS gene expression is detectable within 6 h of induction of NTN, indicating the onset of gene transcription is closely related to the initial formation of immune complexes.

Keywords nitric oxide glomerulonephritis glomerular mesangium kidney polymerase chain reaction

INTRODUCTION

Nitric oxide (NO) is a highly reactive free radical synthesized from L-arginine by a family of nitric oxide synthases (NOS) [1,2]. NO is a vasodilator [3], a neurotransmitter [2], and a mediator of macrophage cytotoxicity against both cells and organisms [1]. There are two major forms of NOS. Constitutive isoforms, which are found in endothelium and in neurones, rapidly synthesize small quantities of NO in response to stimuli which raise intracellular calcium. In contrast, the inducible isoforms of the enzyme (iNOS) are synthesized in response to stimulation by cytokines, and lead to the prolonged production of NO by a calcium-independent process. The inducible enzyme was first identified in murine macrophages [4], but has now been demonstrated in many cells, including smooth muscle cells [5], glomerular mesangial cells [6,7], renal tubular cells [8] and hepatocytes [9,10].

There is increasing evidence that NO is involved in inflammation. In immune complex injury in the lung and skin [11], inhibition of NO synthesis ameliorates the inflammatory response. The presence of iNOS mRNA has been shown in experimental neurological inflammation [12], in the pancreas in experimental diabetes mellitus [13] and in experimental arthritis [14]. The production of NO itself *in vivo* has been shown by the use of electron paramagnetic resonance spectroscopy in allograft rejection [15], septic shock [16] and in experimental allergic encephalomyelitis [17].

In recent studies we have examined the role of NO in glomerular inflammation. We have shown that nephritic glomeruli synthesize NO *in vitro*, as demonstrated by the accumulation of nitrite, a stable end product of NO metabolism, in the culture medium and its inhibition by the specific NOS inhibitor, N^G-monomethyl-L-arginine (L-NMMA). NO synthesis was present in glomeruli from the

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four models of glomerulonephritis we have examined, namely, accelerated nephrotoxic nephritis [18], *in situ* immune complex glomerulonephritis [19], active Heymann nephritis [20] and mesangial proliferative glomerulonephritis induced by antibodies against Thy.1 antigen [21]. However, these results only demonstrate NO synthesis by nephritic glomeruli *in vitro*. The aim of the present study was two-fold. First, to confirm that our previous *in vitro* findings reflected *in vivo* iNOS induction; and second, to relate the onset of induction and the level of gene expression to pathogenic events in immune complex glomerulonephritis. We have used the model of accelerated nephrotoxic nephritis (NTN) in rats, a model where we have previously shown the course of NO synthesis *ex vivo* [18], and where the pathogenesis of injury is known to be dependent on infiltrating macrophages.

MATERIALS AND METHODS

Animals

Inbred male Lewis rats bred at St Mary's Hospital Medical School, London, UK, were used for all experiments. Rats were anaesthetized with halothane (ICI, Macclesfield, UK).

Glomerulonephritis

NTN was induced as previously described [18] by immunization with 1 mg rabbit IgG in Freund's complete adjuvant (FCA) 1 week before an i.v. sub-nephritogenic dose of rabbit anti-rat nephrotoxic serum. Rats were killed at 6 h and 24 h and 2, 4 and 7 days after induction of NTN. Renal biopsies were fixed in formal saline for histological examination. Kidneys were also isolated from normal rats. Glomeruli were isolated by sieving as previously described [18]. In order to preserve the integrity of the glomerular RNA, all isolation steps were performed at 4°C using diethylpyrocarbonate-treated PBS, baked sieves and disposable tissue culture grade plastic. An aliquot was taken for culture for nitrite synthesis and the remaining glomeruli were used immediately for RNA isolation.

Peritoneal cells

Macrophages were isolated from the peritoneal cavity 6 days after i.p. injection of 10 ml thioglycollate broth (Oxoid, Basingstoke, UK) (elicited macrophages) or 10 days after i.p. injection of 0·2 ml *Corynebacterium parvum* (7 mg/ml; Wellcome Laboratories, Beckenham, UK) (activated macrophages). Neutrophils were isolated from the peritoneal cavity 5 h after i.p. injection of oyster glycogen. Peritoneal cells were harvested with PBS at 4°C, washed and aliquoted for cell culture and RNA isolation.

Mesangial cells

Mesangial cells were isolated from 6–10-week-old rats. Glomeruli obtained by sieving were incubated in 750 U/ml collagenase type IV (Sigma, Poole, UK) for 20 min and then cultured in RPMI 1640 (Sigma) plus 2 mg/ml $NaHCO_3$ supplemented with 100 U/ml penicillin, 0·1 mg/ml streptomycin, 0·25 U/ml amphotericin, ITS (5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite) (Sigma) with 20% fetal calf serum (FCS; Globepharm, Esher, UK). Resulting mesangial cell cultures were confirmed by immunostaining with anti-myosin antibody.

Mesangial cells were cultured in the presence or absence of 1 nM recombinant human IL-1β (gift of Glaxo, Geneva, Switzerland) for 17 h before mRNA extraction in 4 M guanidine thiocyanate. Total cellular RNA was purified from the homogenate by caesium chloride density gradient ultracentrifugation.

Reverse transcription polymerase chain reaction

RNA was isolated from glomeruli and peritoneal cells using RNAzol B (Biogenesis, Bournemouth, UK) following the manufacturer's instructions. RNA was quantified by spectrophotometry. Total RNA (10 μg) from each sample was reverse transcribed with Moloney murine leukaemia virus enzyme (Gibco BRL, Paisley, UK) according to the manufacturer's instructions. The cDNA was amplified by polymerase chain reaction (PCR). The PCR primers for iNOS were designed using a published sequence for rat vascular smooth muscle iNOS [5] and were purchased from Oswel DNA services (Edinburgh, UK). For initial detection of iNOS we used primers which amplify a product of predicted length 222 bp. The sense primer was 5′-GCA-TGG-AAC-AGT-ATA-AGG-CAA-ACA-3′ and the antisense primer was 5′-GTT-TCT-GGT-CGA-TGT-CAT-GAG-CAA-3′. At the sites of these primers there is only 38% homology with rat neuronal NOS [22] and the primers would yield a 348-bp product with that molecule. PCR of rat genomic DNA yielded a 1900-bp product, indicating that this primer set spans an intron. For amplification of β actin the primers were 5-TGG-AGA-AGA-GCT-ATG-AGC-TGC-CTG-3′ and 5′-GTG-CCA-CCA-GAC-AGC-ACT-GTG-TTG-3′. PCR was carried out for 30 cycles as described [8], with the modification that an annealing temperature of 63°C was used. PCR products were analysed on 1% agarose gel and visualized with ethidium bromide staining and ultraviolet transillumination.

PCR on samples from peritoneal cells and mesangial cells was carried out using an aliquot of cDNA corresponding to 1·4 μg of total RNA. For glomeruli, in order to obtain a semiquantitative result, PCR using iNOS primers was first carried out using doubling dilutions of cDNA with the highest concentration corresponding to 1·4 μg RNA and the lowest to 89 ng RNA. Having established that there was a semiquantitative relationship between amount of starting template and the intensity of the PCR product, we then estimated the relative amount of iNOS mRNA for each glomerular sample from PCR performed using cDNA corresponding to 178 ng RNA.

Sequencing the PCR products

The 222-bp product of the iNOS PCR reaction was excised from an agarose gel and DNA extracted as described [23]. The fragment was sequenced with the TAQUENCE sequencing kit (Cambridge Biosciences, Cambridge, UK) using a γ^{32}P-ATP-labelled primer (the upstream primer used in the initial PCR reaction).

Competitive PCR

Further quantification of glomerular iNOS mRNA was carried out by competitive PCR using different primers. A deletion mutant (514 bp long) was prepared by PCR using the method of Ho *et al.* [24]. The primer sequences flanking the ends of the wild-type fragment (735 bp long)

were 5′-TCT-TCG-CCA-GAC-CAA-ACT-GTG-TGC-3′ and 5′-GAA-CAA-CAC-TCC-CTT-GCG-GAC-CAT-3′, respectively, for the sense and anti-sense primers. An internal deletion of 221 base pairs was made using internal primers 5′-CAA-CCC-CAC-GTT-CCT-AAG-ACC-CAG-TGC-CCT-3′ and 5′-AGG-GCA-CTG-GGT-CTT-AGG-AAC-GTG-GGG-TGG-3′. The deletion mutant was amplified by PCR using primers complementary to the wild-type fragment and separated from the primers by agarose gel electrophoresis. The mutant-containing band was excised from the gel and the mutant isolated by electroelution. Purified mutant was then quantified by UV spectroscopy, and stock dilutions were prepared for competitive PCR. Competitive PCR was carried out following the method of Peten *et al.* [25]. A master mix was prepared containing standard dNTP and PCR buffer, wild type primers (0·2 μM), and a sample of glomerular cDNA. The master mix was distributed into PCR tubes in 40-μl aliquots, and then 10-μl aliquots of mutant template in decreasing concentrations were added to a series of six tubes. PCR was carried out with an initial incubation at 98°C for 10 min followed by 35 cycles with the following sequential steps: 72°C for 1 min, 58°C for 30 s and 94°C for 30 s. A final incubation was performed at 72°C for 10 min. The PCR products were separated by agarose gel electrophoresis, and the bands visualized by UV illumination of ethidium bromide-stained gels were photographed using Polaroid type 665 positive/negative film. The negatives were scanned by laser densitometry (Chromoscan 3, Joyce Loebl). The densitometric values of the test and mutant bands were measured and their ratio for each tube was plotted against the amount of mutant template added after correction of the mutant density by a factor of 735/514. A straight line was derived by linear regression analysis, and the quantity of cDNA in the test sample was calculated to be that amount at which the mutant/wild-type band density ratio was equal to 1.

Glomerular nitrite production

Glomeruli were cultured at 2000/ml in phenol red free DMEM (Sigma), supplemented with bovine serum albumin (BSA), glutamine, and penicillin and streptomycin. After 48 h supernatants were harvested and nitrite determined by the Griess reaction as described [18].

RESULTS

Rats given nephrotoxic globulin developed a proliferative glomerulonephritis. At 6 h there was glomerular infiltration by mononuclear cells and neutrophils. By 24 h the infiltrate was predominantly mononuclear, and glomerular hypercellularity persisted to 7 days. Nitrite accumulated in the culture medium of nephritic glomeruli and, as in previous experiments [18], synthesis was maximal from glomeruli isolated 24 h after the induction of glomerulonephritis. Nitrite is not detectable in culture supernatants of normal glomeruli. Nitrite production was present in glomeruli isolated at 6 h (4·2 ± 1·5 nmol/2000 glomeruli per 48 h, $n = 3$), maximal at 24 h (21 ± 9 nmol/2000 glomeruli per 48 h, $n = 3$), and still increased above levels in normal glomeruli at 7 days (6 ± 2 nmol/2000 glomeruli per 48 h, $n = 2$).

PCR using iNOS primers demonstrated the expected 222-bp product in samples from activated rat peritoneal macrophages and neutrophils, and from mesangial cells stimulated with IL-1β. No product was found from unstimulated mesangial cells or from peritoneal macrophages elicited with thioglycollate (Fig. 1). A PCR product for iNOS was just detectable in samples from normal glomeruli when PCR was carried out with the highest concentration of starting template, and was present in all nephritic glomeruli.

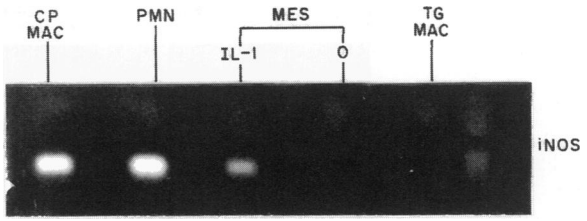

Fig. 1. Reverse transcription polymerase chain reaction (PCR) for inducible nitric oxide synthase (iNOS) in individual cell types. PCR using iNOS primers was carried out on cDNA from isolated cells. In each case the cDNA corresponds to 1·4 μg of total RNA. A PCR product was found in *Corynebacterium parvum*-elicited macrophages (CP MAC), neutrophils (PMN) and IL-1-stimulated mesangial cells (MES) but not in unstimulated mesangial cells (0) or thioglycollate-elicited macrophages (TG MAC).

Sequencing was carried out on iNOS PCR products from glomeruli at 24 h, activated peritoneal macrophages, neutrophils and stimulated mesangial cells. For each sample approximately 80 bp of sequence was read and compared with the published sequence of rat vascular smooth muscle iNOS [5]. The area sequenced corresponded to bp 1801–1881. No significant differences were found, confirming that the products we found originated from iNOS mRNA.

Figure 2 shows a representative experiment in which doubling dilutions of template were used with the 222-bp product primers, and demonstrates that the PCR conditions we chose allow semiquantitative amplification of iNOS cDNA. For comparison of amounts of iNOS cDNA between glomerular samples we carried out PCR using cDNA template derived from 178 ng RNA. All glomerular samples were analysed together in a single PCR run using the same master mix. PCR was also carried out using β actin primers on the same dilutions of glomerular cDNA. The results are shown in

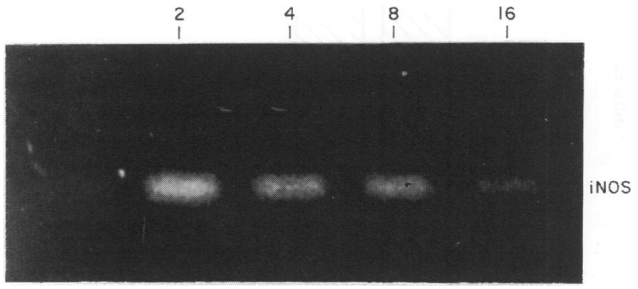

Fig. 2. Relationship between amount of cDNA template and reaction product. Polymerase chain reaction (PCR) for inducible nitric oxide synthase (iNOS) was performed using doubling dilutions of cDNA reverse transcribed from glomeruli isolated 6 h after induction of glomerulonephritis. Lane 2 corresponds to 0·7 μg total RNA.

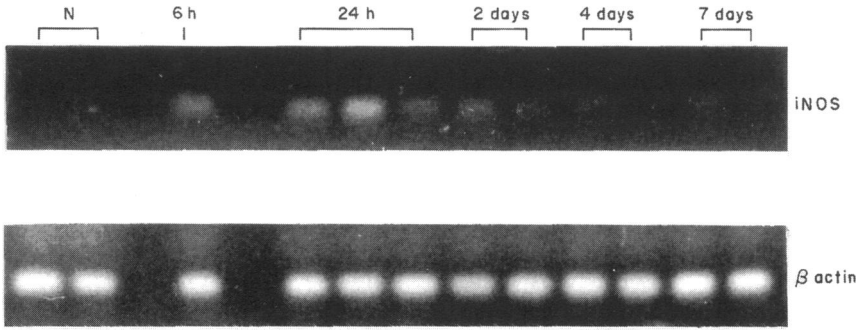

Fig. 3. Time course of inducible nitric oxide synthase (iNOS) expression in nephrotoxic nephritis (NTN). Polymerase chain reaction (PCR) was carried out with iNOS and β actin primers on cDNA from normal glomeruli (N) and glomeruli isolated 6 h and 24 h and 2, 4 and 7 days after induction of glomerulonephritis. PCR was performed with cDNA corresponding to 178 ng RNA.

Fig. 3. There is a clear increase in iNOS mRNA expression by 6 h, and the message appears to be increased compared with normal glomeruli up to 7 days. No difference was seen in the expression of β actin, confirming that the efficiency of reverse transcription did not vary significantly between samples.

For formal quantification of iNOS mRNA we used competitive PCR in which iNOS gave a PCR product of 735 bp; a PCR product of the expected length was found in each of the glomerular samples. Figure 4 shows the results of competitive PCR. The results are expressed as attomoles of iNOS cDNA present in the reverse transcription product of 10 μg of total glomerular RNA. These results confirmed that there was a marked rise in iNOS mRNA by 6 h from 2 ± 1 attomoles to 863 attomoles, and that levels remained elevated up to 7 days.

DISCUSSION

This study demonstrates that normal rat glomeruli express low levels of the gene for iNOS and that glomeruli in immune complex glomerulonephritis show increased expression of this gene. The peak of expression at 24 h corresponds to the time at which *ex vivo* production of nitrite is highest.

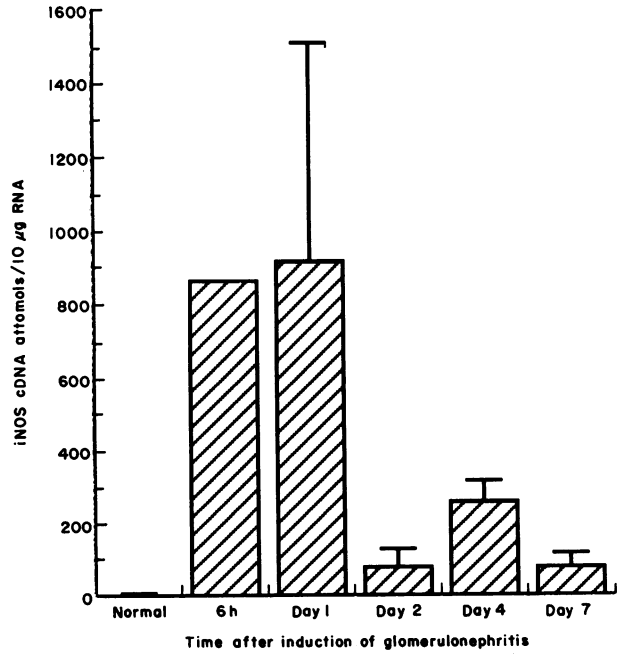

Fig. 4. Levels of cDNA corresponding to inducible nitric oxide synthase (iNOS) mRNA in normal and nephritic glomeruli as determined by competitive polymerase chain reaction (PCR).

These findings confirm that our previous *ex vivo* studies of NO synthesis in nephritic glomeruli do indeed reflect *in vivo* iNOS induction. They are complementary to recent studies, in which we have demonstrated iNOS by immunohistochemistry in another model of immune complex glomerulonephritis [26]. It is of importance to study both gene and protein expression, since detailed studies on mouse macrophages have shown that expression of iNOS may be controlled at multiple levels [27]. In that study transforming growth factor-beta (TGF-β) led to a reduction in iNOS protein by enhancing degradation of iNOS mRNA, by impairing its translation, and by increasing the rate of protein degradation.

In nephrotoxic nephritis there is rapid fixation of antibody along the glomerular capillary wall, followed by an early influx of both neutrophils and macrophages and glomerular injury as reflected by proteinuria. By 24 h macrophages are the main inflammatory cell and increased macrophage numbers persist up to 7 days. Proteinuria after the first 24 h is macrophage-dependent [28]. We found that iNOS expression increased by 6 h, and this is consistent with studies on macrophages [27,29] showing induction of iNOS mRNA by 6 h after stimulation, studies on neutrophils [30] showing maximal NOS activity 6 h after elicitation, and studies on mesangial cells showing NO-dependent cGMP formation 4–8 h after cytokine stimulation [6]. After a single stimulus macrophage iNOS expression is undetectable by 48 h [29], and so our finding of continuing expression up to 7 days implies a continuing stimulus and possibly continuing recruitment of cells to the glomerulus, which are then induced to express iNOS. iNOS expression was just detectable in normal glomeruli using the highest amount of starting template, indicating that there may be a continuing small stimulus to iNOS induction even in the normal animal; a possible source is the resident glomerular macrophage [31]. This raises the possibility that NO synthesized by the inducible enzyme may be of importance in control of haemodynamics in the normal glomerulus.

The stimulus to iNOS induction in glomerulonephritis is of some interest. *In vitro* macrophages are stimulated to synthesize NO by cytokines such as interferon-gamma (IFN-γ) [32] and mesangial cells can be stimulated by IL-1β or tumour necrosis

factor-alpha (TNF-α) [6]. In addition, phagocytosis is able to provide a co-signal [33]. In nephrotoxic nephritis there is rapid induction of IL-1β [34] and TNF-α [35], and both mesangial cells and macrophages may phagocytose immune complexes. Definite identification of the stimuli to iNOS induction will require further experiments with specific inhibitors.

In order to determine whether our method could detect iNOS induction in individual cell types in the inflamed glomerulus, we also studied the expression of iNOS in macrophages, neutrophils and mesangial cells. As expected, macrophages obtained from the peritoneum after *C. parvum*, a potent macrophage activator, showed strong expression of iNOS, whereas no iNOS was detected in macrophages elicited with thioglycollate. This is consistent with studies showing that thioglycollate macrophages require a further stimulus to induce NO synthesis [32]. We found that neutrophils isolated 5 h after oyster glocogen, a time when NOS activity is reported as high [30], also expressed a similar iNOS gene, and thus it is likely that neutrophils may contribute to the iNOS expression we found at 6 h in NTN.

We and others [36] have shown that mesangial cells stimulated with IL-1β synthesize NO, and our present results show that the mesangial cell gene is closely related to that in macrophages and neutrophils. Indeed, it is likely that the gene in these three cell types and in vascular smooth muscle cells and hepatocytes [37] are identical. Recent gene sequence data from human cells also suggest that there is only a single gene for inducible NOS [38]. Thus our results do not allow us to discriminate between mesangial cell or macrophage expression of iNOS in nephritic glomeruli. However, in our previous studies we found that *ex vivo* nitrite production was closely related to macrophage influx [19], and that whole body irradiation suppressed nitrite production and macrophage infiltration [20]. In this study the peak of iNOS expression corresponded with the major macrophage influx into glomeruli as judged by light microscopy.

Since our initial studies in isolated glomeruli [18], which were the first demonstration of NO production at a site of immunologically mediated inflammation, NO synthesis has been shown in immune complex injury in lung [11], skin [11], the central nervous system (CNS) [12] and joints [14]. In the CNS and joints increased expression of iNOS mRNA has been demonstrated. Inhibition of NOS has been shown to ameliorate immune complex injury in both the lung [11] and joints [14]. The present study provides further evidence consistent with a role for NO in immune complex-induced disease in the renal glomerulus. An important finding which contributes towards the understanding of the regulation of the enzyme in this type of disease is that the onset of induction of iNOS is very closely related to the initial formation of immune complexes.

ACKNOWLEDGMENTS

This work was supported by grants from the Medical Research Council and The Wellcome Trust. Dr Chih-Wei Yang, National Institutes of Health, Bethesda, MD, provided invaluable advice on the methodology of the competitive PCR reaction.

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