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Glomeruli synthesize nitrite in active Heymann nephritis; the source is infiltrating macrophages

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Glomeruli synthesize nitrite in active Heymann nephritis; the source is infiltrating macrophages. Glomeruli synthesize nitrite (NO_2^-) in experimental nephrotoxic nephritis, a model of glomerulonephritis where infiltrating macrophages are pathogenic. NO_2^- synthesis was studied in active Heymann nephritis (AHN), a model of membranous glomerulonephritis in which macrophages have not been implicated. Active Heymann nephritis (AHN) was induced with purified renal tubular epithelial antigen and adjuvants. Glomeruli isolated at seven to eight weeks after induction (proteinuria 183 ± 28 mg/24 hr, $N = 6$; adjuvant controls, 1.2 ± 0.8 mg/24 hr, $N = 6$) produced NO_2^- in culture spontaneously (7.1 ± 1.4, adjuvant controls 2.1 ± 0.9 nmol/2000 g/48 hours; $P = 0.021$) and in increased amount following LPS stimulation (12.1 ± 2.8, controls 4.2 ± 1.6 nmol/2000 g/48 hours; $P = 0.047$). Synthesis was inhibited by L-NMMA, a competitive inhibitor of NO synthase. Enzymic digestion of glomeruli plus staining with mouse anti-rat macrophage monoclonal antibody ED1 showed macrophage infiltration (32 ± 6, adjuvant controls 14 ± 2 macrophages/glomerulus; $P = 0.002$). Whole body irradiation (XR) suppressed NO_2^- production (LPS stimulated: 1.0 ± 0.4, $N = 5$; non-XR controls 7.2 ± 4.6 nmol/2000 g/48 hours; $N = 5$, $P = 0.016$) and macrophage infiltration (1.1 ± 0.5; non-XR controls 30 ± 12 macrophages/glomerulus; $P = 0.008$) but had no effect on proteinuria. Irradiation with renal shielding confirmed the close correlation between glomerular NO_2^- synthesis and glomerular macrophage numbers ($r_s = 0.837$, $P < 0.001$). These results show that macrophages infiltrate glomeruli in AHN; they are the source of NO_2^- in this model. Neither macrophages nor NO_2^- are the cause of proteinuria.

We have recently shown that glomeruli isolated from experimental nephrotoxic nephritis synthesize nitrite (NO_2^-), a stable product of the L-arginine-nitric oxide (NO) pathway [1]. This synthesis was spontaneous and further stimulated by lipopolysaccharide (LPS). As we were unable to detect NO_2^- production by normal glomeruli, it seemed likely that synthesis was induced by immunological or inflammatory events in glomerulonephritis, and therefore might be important in pathogenesis.

In nephrotoxic nephritis macrophage infiltration causes glomerular hypercellularity and injury [2]. Our results in this model showed that peak NO_2^- production coincided with the main influx of macrophages [1]. This suggested that macrophages, which are known to produce NO and NO_2^- on activation [3] were the source of glomerular NO_2^-. However, the inducible form of the enzyme NO synthase which generates NO has now been identified not only in macrophages but in increasingly diverse cell types, including endothelium which initially only appeared to express constitutive NO synthase [4]. In addition, glomerular mesangial cells show evidence of activation of the NO pathway on stimulation with LPS or cytokines [5, 6]. It has therefore become important in investigating the role of NO in glomerulonephritis to identify the cellular source.

We studied active Heymann nephritis (AHN) [7], a model of immune complex-induced glomerulonephritis in which macrophages have not been implicated, to determine whether NO_2^- is generated in this type of immune injury, and if so its source and role in proteinuria.

Methods

Materials

Glomerular culture was carried out in Dulbecco's modified Eagle's medium without phenol red (Sigma Chemical Co., Poole, UK) supplemented with L-glutamine (584 mg/liter) and 10% fetal calf serum (Flow Laboratories, Irvine, UK). Polymyxin B (Sigma Chemical Co.) 10 μg/ml was added to all washing buffers for glomerular isolation in all experiments. LPS (*E. coli* O127:B8, Sigma) and N^G-monomethyl-L-arginine (L-NMMA; Wellcome Research Laboratories, Beckenham, UK) were diluted in medium for use in glomerular incubations. Griess reagent for NO_2^- assay was sulfanilamide 1% (Sigma), naphthyl ethylenediamine dihydrochloride 0.1% (Sigma) in 2.5% orthophosphoric acid.

Rats

Inbred female Lewis rats from St. Mary's Hospital Medical School were used.

Glomerular isolation

Glomeruli were isolated from kidneys perfused in vivo with 50 ml sterile pyrogen-free saline (Phoenix Pharmaceuticals, Gloucester, UK) at room temperature as previously described [8]. They were washed twice in glomerular isolation buffer before use.

Leucocyte quantitation in whole glomeruli

Leucocytes were enumerated in whole glomeruli by labelling with mouse monoclonal antibody to rat leucocyte common

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antigen [9] (clone OX1, Seralab, Crawley Down, UK). This method was used to confirm that the dose of whole body irradiation caused glomerular leucocyte depletion (see *Irradiation protocol*). Ia expressing cells in whole glomeruli were identified with monoclonal antibodies OX3 and OX4 (Seralab) as described [9].

Macrophage isolation from nephritic glomeruli

Isolated glomeruli were enzymically digested to single cell suspensions using trypsin, collagenase, DNase and EDTA (Sigma) as described [10]. The total cell number isolated was recorded, cytospin preparations were fixed in acetone and stained with monoclonal mouse anti-rat macrophage antibody ED1 as described [11].

NO_2^- production in vitro and assay

Isolated glomeruli were plated at 2000/ml in 16 mm plastic tissue culture wells (Nunc, Uxbridge, UK), and incubated for 48 hours at 37°C under 4% CO_2, with or without LPS 1 μg/ml or L-NMMA 300 μM. Supernatants were collected and stored at −20°C before assay.

NO_2^- concentration was determined by the Griess reaction as previously described [1]. Values were derived by subtracting background NO_2^- present in medium incubated for 48 hours without glomeruli.

Glomerulonephritis

Active Heymann nephritis was induced in 7-week-old female Lewis rats as previously described [12]. Purified renal tubular antigen (RTE) 2.5 mg and 0.5 mg lyophilized M. tuberculosis H37 RA (Difco, Detroit, Michigan, USA) in 100 μl Freund's incomplete adjuvant was injected into each front footpad. Bordetella pertussis (Difco) 5×10^8 organisms was injected into the dorsum of each front footpad. Rats received tetracycline hydrochloride (Lederle, Gosport, UK) 0.2 g/liter in drinking water. Twenty-four hour urine collections were performed weekly and at other times where indicated, and protein determined by the sulphosalicyclic acid method. In some experiments control rats receiving identical immunization with adjuvants but without RTE were used.

Histology, immunohistochemistry and electron microscopy

Kidney tissue for histology was fixed in formal saline. Paraffin sections were stained with hematoxylin and eosin and periodic acid Schiff. Immunostaining for rat IgG was performed as previously described [8]. Tissue for electron microscopy was fixed in 4% buffered glutaraldehyde at 4°C and processed and examined as previously described [8].

Irradiation

Rats received 10 Gy (Siemens Stabilipan X-ray machine 10 mA, 240 kV, 0.48 Gy/min) either whole body irradiation or with renal shielding (0.5 cm thick lead cuff protecting both kidneys) Neomycin (100 mg/liter) and polymixin (10 mg/liter) were given in drinking water from three days prior to irradiation until the end of the experiment at four days post-irradiation, to protect against possible infection [9].

Experimental protocol

In the first experiment glomeruli were isolated from AHN rats and controls receiving adjuvants without RTE at seven and eight weeks after induction of glomerulonephritis. Glomeruli were isolated for macrophage quantitation and incubated for NO_2^- production. Tissue was examined by light, immuno- and electron microscopy.

In irradiation experiments initial whole body irradiation was performed on normal rats to establish a dose producing glomerular macrophage depletion in female Lewis rats and to determine any effect on NO_2^- production. Then whole body irradiation was given to AHN rats seven weeks after induction of disease and to a group of normal rats. A third group of non-irradiated AHN rats were also studied in this experiment. Glomerular isolation was performed four days after irradiation. Urinary proteins were estimated one day prior to irradiation and in the final 24 hours before sacrifice. In a final irradiation experiment, the above experiment was repeated in four rats at eight weeks after induction of AHN using 10 Gy with renal shielding.

Statistics

Results are expressed as mean ± standard error of the mean. Comparisons between groups are by Mann-Whitney U test and Spearman rank correlation coefficient.

Results

The first experiment examined the course of AHN, glomerular NO_2^- synthesis and macrophage infiltration.

Proteinuria was first detected at four weeks (8.5 ± 3.0 mg/24 hr; $N = 5$) and rose to 183 ± 28 mg/24 hr at seven weeks ($N = 6$). Controls immunized with adjuvant alone 1.2 ± 0.8 mg/24 hr ($N = 6$).

By light microscopy kidneys from AHN rats sacrificed at seven to eight weeks showed no glomerular changes apart from some prominence of visceral epithelial cells. There were some homogeneous, eosinophilic tubular casts. Granular capillary wall deposits of rat IgG were found by immunohistochemistry (Fig. 1). By electron microscopy there were multiple small discrete subepithelial deposits resembling human membranous glomerulonephritis (Fig. 2). No immune deposits were detected in control rats.

Glomeruli from AHN rats synthesised NO_2^- basally; production was stimulated by LPS and inhibited by L-NMMA (Table 1). NO_2^- synthesis was significantly higher than in control rats immunized with adjuvant alone both basally ($P = 0.021$) and with LPS ($P = 0.047$). The control rats, however, produced some detectable NO_2^- not previously found in normal male Lewis rats [1].

Quantification of glomerular macrophages (Table 1) showed an increase in AHN rats compared with adjuvant control rats ($P = 0.002$) in which glomerular macrophage numbers were not significantly different from those found in normal male Lewis rats [11]. In normal rat glomeruli 47 ± 10% of glomerular macrophages expressed Ia antigen while in AHN rats 17 ± 1% were Ia positive. This suggests that most of the infiltrating macrophages in AHN are not expressing Ia.

In irradiation experiments the following results were obtained: in normal rats 10 Gy whole body irradiation did not

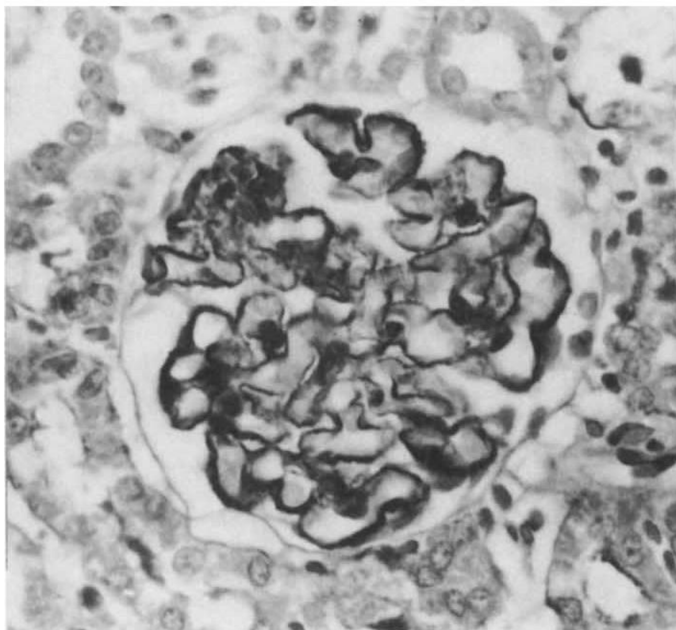

Fig. 1. *Granular capillary wall deposits of rat IgG in glomerulus from AHN rat at 7 weeks.* Immunoperoxidase rabbit anti-rat IgG. ×480

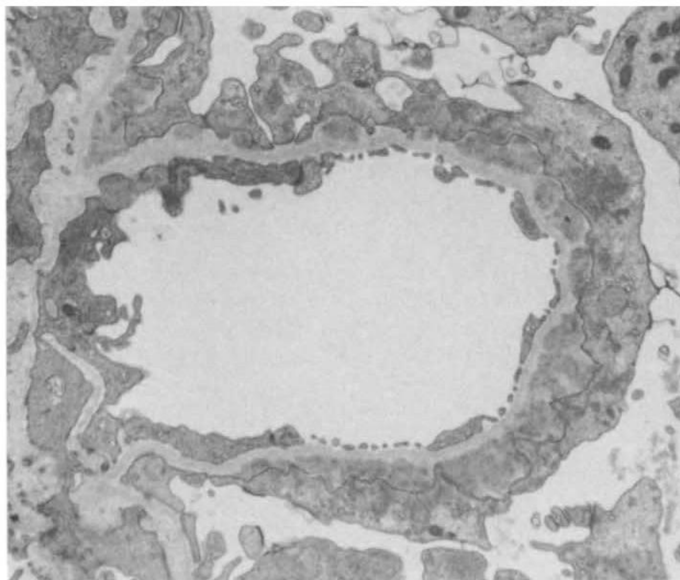

Fig. 2. *Glomerular capillary loop in AHN at 7 weeks.* There are multiple small subepithelial electron dense deposits. ×8300

stimulate significant NO_2^- production; glomerular leucocytes were depleted (0.6 ± 0.1 leucocytes/glomerulus, $N = 4$; unirradiated controls 13.5 ± 1.4, $N = 2$). This protocol was therefore used in AHN to determine the contribution of macrophages to NO_2^- synthesis.

In rats with AHN which received whole body irradiation (Table 2) glomerular NO_2^- synthesis was suppressed both basally ($P = 0.048$) and with LPS ($P = 0.016$) and macrophage infiltration abolished ($P = 0.008$) compared with AHN rats in the same cohort which did not receive irradiation. As above, irradiation in normal rats caused macrophage depletion but no stimulation of NO_2^- synthesis. In AHN irradiation did not significantly affect proteinuria.

To confirm that the effect of irradiation on NO_2^- was due to the observed depletion of macrophages and not a direct effect on irradiation on intrinsic glomerular cells an irradiation experiment with renal shielding was performed. Although macrophage depletion was not as complete as with whole body irradiation, the quantity of NO_2^- produced after LPS stimulation was directly related to the numbers of macrophages per glomerulus (Fig. 3). The data from the whole body irradiation experiment and the experiment with renal shielding were analyzed together, and confirmed this close correlation between glomerular NO_2^- production and glomerular macrophage numbers ($r_s = 0.837$, $P < 0.001$ for LPS stimulated production and $r_s = 0.728$, $P = 0.004$ for basal production; $N = 14$). In contrast there was no correlation between glomerular macrophage numbers and proteinuria ($r_s = -0.018$, NS).

Discussion

The active form of Heymann nephritis induced in these experiments was as previously described by others [12]. We found an increasing proteinuria starting at four weeks, a membranous form of glomerulonephritis with capillary wall deposits of rat IgG and multiple subepithelial electron dense deposits by electron microscopy.

Glomeruli isolated from nephritic kidneys at seven to eight weeks produced NO_2^- basally, enhanced by the addition of 1 μg/ml LPS. Synthesis was inhibited by L-NMMA a competitive inhibitor of NO synthesis. Levels of NO_2^- were not as high as those we have previously found in accelerated nephrotoxic nephritis [1]. We also found detectable, but significantly lower production in the control rats immunised with adjuvants alone; this suggests some induction of enzyme due to activation of intrinsic glomerular macrophages.

Contrary to previous reports, we have demonstrated macrophage infiltration in AHN glomeruli; this is almost certainly because the methods we used to quantitate this infiltration have not previously been applied to AHN. We used this method in active in situ glomerulonephritis [11] and accelerated nephrotoxic nephritis [13], and have shown it to be sensitive and reproducible. The numbers of macrophages in AHN are less than in the above models, which may account for failure to detect them morphologically as the glomeruli do not have a hypercellular appearance. The number of glomerular macrophages in adjuvant controls was no different from normal male Lewis rats [11].

The unpredicted finding of NO_2^- generation in this model led to further experiments to determine the source of NO_2^-. There were two possible sources, either the infiltrating macrophages we had detected or an intrinsic glomerular cell such as the mesangial cell, which has been shown in vitro to produce NO_2^- with certain stimuli [5, 14]. We therefore studied NO_2^- production in AHN following macrophage depletion. Whole body irradiation, resulting in macrophage depletion in glomeruli, entirely suppressed NO_2^- synthesis in AHN. This showed that

Table 1. NO_2^- synthesis and macrophage infiltration in AHN

	N	NO_2^- *nmol/2000 g/48 hr* Basal	L-NMMA	LPS	LPS + L-NMMA	Macrophages/g
AHN	6	7.1 ± 1.4[a]	1.6 ± 0.7	12.1 ± 2.8[b]	3.2 ± 1.7	32 ± 6[c]
Adjuvant controls	6	2.1 ± 0.9	1.3 ± 0.6	4.2 ± 1.6	1.5 ± 0.7	14 ± 2

[a] $P = 0.021$
[b] $P = 0.047$
[c] $P = 0.002$

Table 2. Effect of whole body irradiation in AHN

	N	NO_2^- *nmol/2000 g/48 hr* Basal	L-NMMA	LPS	LPS + L-NMMA	Macrophages/g	Urine protein[d] *mg/24 hr* Pre	Post
Irradiated AHN	5	0.9 ± 0.3[a]	0.2 ± 0.1	1.0 ± 0.4[b]	0.5 ± 0.1	1.1 ± 0.5[c]	21 ± 11.0	25 ± 16.5
AHN	5	2.6 ± 1	0.8 ± 0.2	7.2 ± 4.6	0.6 ± 0.3	30 ± 12	30 ± 18	43 ± 20.4
Irradiated normal rats	3	0.8 ± 0.1	0.9 ± 0.3	0.7 ± 0.1	0.7 ± 0.1	1 ± 0.4	1.3 ± 0.3	2

[a] $P = 0.048$
[b] $P = 0.016$
[c] $P = 0.008$
[d] Urinary protein measured 24 hours pre- and 3 days post-irradiation

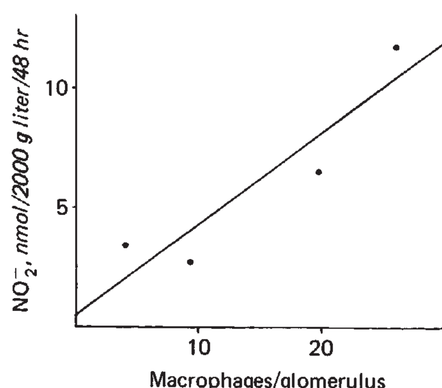

Fig. 3. *Effect of irradiation with renal shielding on infiltrating macrophage numbers and NO_2^- synthesis.*

mesangial cells were not the source of NO_2^- in the absence of macrophage infiltration. To ensure that this was not an effect of irradiation on intrinsic glomerular cells, we also performed irradiation with renal shielding. The experiments clearly showed a direct relationship between NO_2^- suppression and inhibition of infiltrating macrophages. Overall we found a highly significant correlation between NO_2^- production and glomerular macrophage numbers in active Heymann nephritis. A further point of interest is that the experiments also show that NO_2^- is not generated by glomeruli in response to capillary wall antibody and complement deposition alone.

Our data is specific for AHN and it is possible that there may be instances in vivo where mesangial cells could contribute to glomerular NO_2^- synthesis. However, we have been unable to detect any NO_2^- synthesis by whole glomeruli after depletion of resident macrophages even with doses of LPS as high as 100 μg/ml and after stimulation of whole glomeruli with IL-1 (unpublished observations).

Irradiation did not lead to any reduction in proteinuria, confirming that the macrophage is not the cause of proteinuria in this model (although strictly speaking these results only have a bearing on the maintenance of proteinuria and not its induction) and supporting other studies indicating complement-mediated injury [15] similar to that in the passive form of Heymann nephritis [16].

These experiments also indicate that NO_2^- production, at this level, is not the cause of proteinuria. As there is in vitro evidence that NO is cytotoxic [17], it is possible that NO_2^- generated in AHN is not directly injurious because a cytotoxic level is not achieved. We also cannot exclude a possible effect on glomerular hemodynamics, as this was not studied in our current experiments. It is clear from other studies that inhibition of NO affects renal hemodynamics [18], and there is increasing investigation of the role of NO in renal physiology.

In conclusion, we find that glomeruli in AHN synthesize NO_2^- when cultured ex vivo. This synthesis is due to infiltrating macrophages, which have not previously been detected in this model. These findings in AHN strongly suggest that the NO_2^- we have documented in accelerated nephrotoxic nephritis [1] is generated by infiltrating macrophages. Although in AHN neither NO_2^- synthesis nor macrophages are implicated in the production of proteinuria, which remains most probably of complement mediated origin, it seems likely that in the former model, which is macrophage dependent, NO may be pathogenic.

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