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Glomerular Nitrite Synthesis in *In Situ* Immune Complex Glomerulonephritis in the Rat

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Nitrite (NO_2^-) is the major end product of nitric oxide (NO) production in cell culture. The authors have examined nitrite production by glomeruli in* in situ *immune complex glomerulonephritis in the rat. Glomerulonephritis was induced by unilateral renal perfusion of cationized human gamma G immunoglobulin (IgG) in preimmunized rats. NO_2^- was measured in culture supernatants of isolated glomeruli after 48 hours. NO_2^- was produced by nephritic glomeruli with a maximum 4 days after induction of glomerulonephritis (24.4 ± 11.4 pmol/glomerulus/48 hours). Production was increased by lipopolysaccharide (LPS; 1 μg/ml) (54 ± 4.9 pmol/glomerulus; P < 0.001). NO_2^- production was inhibited by the nitric oxide synthase inhibitor N^G-monomethyl-L-arginine demonstrating synthesis through NO. Dexamethasone (10^{-7} mol/l [molar]) reduced LPS-stimulated production by peritoneal macrophages and nephritic glomeruli (P < 0.01). Macrophages isolated from nephritic glomeruli produced NO_2^- (4.9 ± 0.6 nmols/10^5 cells). The production of NO by nephritic glomeruli has implications for mechanisms of glomerular injury and glomerular hemodynamics. The effect of dexamethasone may explain in part the ameliorative effect of steroids in glomerulonephritis. (Am J Pathol 1991, 139:1047–1052)

The synthesis of nitric oxide (NO) from L-arginine was originally described in endothelial cells[1] and has now been described in many other cells and tissues.[2–7] Nitric oxide is synthesized by activated macrophages,[8–12] and we have previously shown[13] synthesis by glomeruli in nephrotoxic nephritis, a form of macrophage-dependent glomerular injury. We have now studied the synthesis of nitrite (NO_2^-), the major stable end product of NO in culture, in a model of unilateral *in situ* glomerulonephritis and examined the effects of dexamethasone on macrophage and glomerular synthesis of NO.

Materials and Methods

Animals

Inbred male Lewis rats from St. Mary's Hospital Medical School were used for all experiments. Operative procedures were carried out with halothane (May & Baker, Dagenham, UK) anesthesia.

Induction of Glomerulonephritis

Glomerulonephritis was induced as previously described.[14] Briefly, rats were immunized three times with human gamma G immunoglobulin (IgG) in Freund's complete adjuvant and then the left kidney was perfused with 400 μg cationized IgG.

Isolation of Peritoneal Macrophages

Peritoneal macrophages were obtained by peritoneal lavage with calcium-free perfusion buffer (pH 7.4) from rats injected intraperitoneally 6 days previously with thioglycollate broth (Difco, East Molesey, UK)

Glomerular and Cell Culture

Culture was carried out in Dulbecco's modified Eagles medium (DMEM; Sigma, Poole, UK), without phenol red, supplemented with glutamine (584 mg/l), penicillin (50 units/ml), streptomycin (50 μg/ml), and 10% fetal calf serum (FCS; Flow Laboratories, Wycombe, UK). The concentration of L-arginine in this medium is 0.5 mmol/l (mil-

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limolar). All glassware was baked at 170°C for 4 hours to destroy endotoxin. Polymyxin B (Sigma), 10 mg/l, was added to all washing buffers for glomerular isolation. The fetal calf serum contained 0.12 ng/ml endotoxin, as determined by the supplier.

Glomeruli were isolated from kidneys by differential sieving as previously described.[15] Isolations were carried out on normal rats, preimmunized rats, and at 1, 2, 4, and 7 days after the induction of glomerulonephritis. Glomeruli were plated at 2000/ml in DMEM + FCS in 16-mm tissue culture wells (Nunc, Uxbridge, UK) and incubated for 48 hours at 37°C under 4% CO_2. Lipopolysaccharide (LPS; 1 μg/ml [*Escherichia coli* 055:B5, Sigma]), N^G-monomethyl-L-arginine (L-NMMA; Wellcome Research Laboratories, Beckenham, UK), L-arginine, D-arginine, or dexamethasone (Sigma) were added to some wells. Dexamethasone was dissolved at 20 mg/ml in ethanol and further diluted in DMEM + FCS.

Peritoneal macrophages were plated at 1×10^6/ml in 1 ml DMEM + FCS in 16-mm wells and washed after 2 hours to remove nonadherent cells. They then were cultured for 48 hours, and NO_2^- was measured in supernatants. Cell viability was assessed as described in macrophages adhered to glass coverslips.[16]

In one experiment, isolated glomeruli were enzymatically digested to single-cell suspensions using trypsin, collagenase, and DNase.[17] Cells were plated at 1.5×10^6/ml and washed after 2 hours to provide a population of glomerular macrophages, as previously described.[14]

Nitrite Assay

Nitrite in culture supernatants was measured at 24 and 48 hours of cell or glomerular culture by the Griess reaction.[10] One hundred microliters supernatant was mixed with an equal volume of Griess reagent in a 96-well plate, and the absorbance was read at 550 nm (Titertek, Multiscan Plate Reader, Flow laboratories). Nitrite concentration was determined using sodium nitrite as standard. Values were derived by subtracting background NO_2^- present in medium incubated without glomeruli for 48 hours. The lower limit of detection was 1.5 nmol/ml. Results are expressed as pmol/glomerulus obtained by dividing the amount of NO_2^- generated by 2000, the number of glomeruli initially plated.

Statistics

All comparisons are by Student's test. Results are shown as mean ± standard deviation.

Results

In Situ *Glomerulonephritis*

The histology and glomerular cell infiltration in this model has been described before.[14] Briefly, no abnormalities were seen by light microscopy in right kidneys. Left kidneys developed glomerular hypercellularity, with 381 ± 64 macrophages/glomerulus and 165 ± 21 neutrophils/glomerulus at day 4. The Ia expression of glomerular macrophages increased from 15 ± 3% at day 1 to 42 ± 4% at day 4.

Glomerular Nitrite Production

No NO_2^- was detected from glomeruli from normal rats (n = 5) or from rats preimmunized with human IgG and Freund's complete adjuvant (n = 5) under either basal conditions or when incubated with LPS (1 μg/ml).

Table 1 shows NO_2^- production after 48 hours' culture from left and right kidneys isolated at various times after induction of glomerulonephritis. At 2, 4, and 7 days, basal production was significantly greater in left nephritic

Table 1. *Nitrite Production by Glomeruli Isolated at Various Time Points after Induction of Glomerulonephritis*

	Nitrite production pmol/glomerulus			
	Day 1	Day 2	Day 4	Day 7
Left kidney				
Basal	2.5 ± 2.4	18.7 ± 6.9*	24.4 ± 11.4†	8.8 ± 2.7*
+LPS	3.2 ± 2.9	33.1 ± 3.6‡	54.0 ± 4.9‡	24.3 ± 3.0‡
Right kidney				
Basal	1.6 ± 1.5	0.8 ± 0.5	0.7 ± 0.7	4.5 ± 0.5
+LPS	0.2 ± 0.3	4.2 ± 4.7	0.8 ± 1.1	4.1 ± 0.2

* $P < 0.05$ vs. right kidney.
† $P < 0.001$ vs. right kidney.
‡ $P < 0.001$ vs. basal.
Incubation was carried out under basal conditions or with LPS 1 μg/ml (left kidneys n = 6; right kidneys n = 3).

glomeruli than in glomeruli from control right kidneys ($P <$ 0.05 days 2 and 7; $P < 0.001$ day 4). Production was maximal at 4 days (24.5 ± 11.4 pmol/glomerulus/48 hours). At 2, 4, and 7 days, NO_2^- was significantly increased by LPS ($P < 0.001$). Control right kidneys produced small but detectable amounts of NO_2^-.

Table 2 shows the effect of L-NMMA on NO_2^- production by LPS-stimulated nephritic glomeruli. There was marked inhibition of NO_2^- at all times studied. The inhibition of NO_2^- production by glomeruli isolated 4 days after induction of glomerulonephritis was inhibited in a dose-dependent fashion by L-NMMA, and inhibition was reversed by 4.2 mmol/l L-arginine (Figure 1), but not by 4.2 mmol/l D-arginine.

In most experiments, glomerular supernatants also were sampled for NO_2^- after 24 hours of incubation, and in general the quantities of NO_2^- were approximately half those at 48 hours, indicating that synthesis occurs in an approximately linear fashion over 48 hours, as previously reported.[13] This also suggests that it is unlikely that there is any appreciable loss of viability of the cells producing NO_2^- during the later part of the incubation.

Effect of Dexamethasone on Production of Nitrite

Thioglycollate-elicited peritoneal macrophages produced small amounts of NO_2^- under basal conditions, and production was markedly enhanced by LPS stimulation. Dexamethasone inhibited NO_2^- production (Figure 2) when added 2 hours before LPS. In separate experiments, inhibition still occurred when dexamethasone was added at the same time as LPS (results not shown). Ethanol, which was used to dissolve dexamethasone, had no effect on NO_2^- production at appropriate concentrations. Dexamethasone had no effect on macrophage viability at 10^{-5} mol/l (99% viability), but there was some reduction at 10^{-3} mol/l (78%).

Dexamethasone caused a reduction in LPS-stimulated NO_2^- production from glomeruli isolated 4 days after the induction of glomerulonephritis (Figure 3) ($P < 0.01$) at 10^{-7} mol/l dexamethasone and higher concentrations. In addition, examination of plated glomeruli showed that increasing concentrations of dexamethasone inhibited macrophage outgrowth from nephritic glomeruli (Figure 4).

Macrophages were isolated from nephritic glomeruli 4 days after induction of glomerulonephritis by enzyme digestion. We recovered 168 ± 37 macrophages/glomerulus (n = 3). NO_2^- generation per 10^5 macrophages plated was 4.9 ± 0.6 nmols (L-NMMA 0.26 ± 0.03 nmols), and with LPS stimulation, 10.5 ± 0.7 nmols/10^5 cells.

Discussion

Nitric oxide is synthesized in mammalian cells from the terminal guanido nitrogens of L-arginine with the concomitant generation of L-citrulline.[11,18] N^G-monomethyl-L-arginine is a competitive inhibitor of this pathway. In culture, the stable end products of nitric oxide are nitrite and nitrate, and thus the production of nitrite, which is inhibitable by L-NMMA, provides a measure of NO synthesis.[9] In this model of *in situ* immune complex glomerulonephritis, glomeruli produce nitrite spontaneously in culture with maximum production 4 days after induction of glomerulonephritis.

Nitrite production was inhibited in a dose-dependent fashion by L-NMMA, and this inhibition was reversed by L-arginine but not by D-arginine, demonstrating that it was synthesized through NO. The amount of L-arginine needed to reverse the effect of L-NMMA is large, but this is similar to the results other investigators have found.[19] Non-nephritic right kidneys produced only small amounts of NO_2^-, thus indicating that the production by nephritic glomeruli was due to local, rather than systemic, activation of NO synthesis.

No NO_2^- was produced by glomeruli from normal rats or rats that had been preimmunized with human IgG and Freund's complete adjuvant. The presence of small, but detectable, synthesis by right kidneys, even in the absence of detectable histologic changes, was unexpected. There are three possible explanations. Firstly it may be due to small amounts of cationized antigen moving from the original site of fixation in the left kidney and depositing in the right kidney with a low-grade glomeru-

Table 2. *Effect of L-NMMA on Nitrite Production by LPS-Stimulated Nephritic Glomeruli*

	Nitrite production pmol/glomerulus			
	Day 1	Day 2	Day 4	Day 7
LPS	3.2 ± 2.9	33.1 ± 3.6	54.0 ± 4.9	24.3 ± 3.0
LPS + L-NMMA	0.2 ± 0.2*	2.2 ± 0.6*	2.5 ± 2.2*	4.1 ± 0.4*

Glomeruli were incubated with LPS 1 μg/ml ± L-NMMA 300 μM.
* $P < 0.001$.

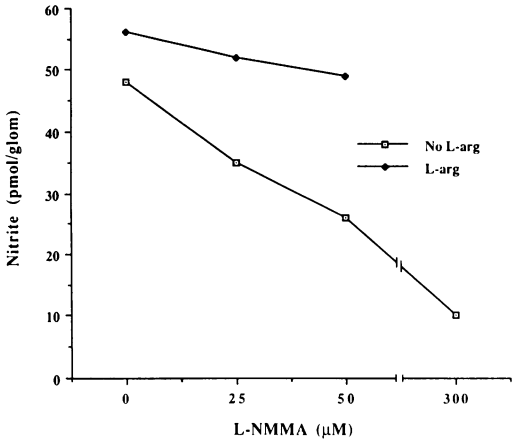

Figure 1. *Effect of various concentrations of L-NMMA on nitrite generation by glomeruli in the presence or absence of added L-arginine. Glomeruli were isolated 4 days after induction of glomerulonephritis and incubated for 48 hours with no added L-arginine or with L-arginine 4.2 mM added to the medium. Each point is the mean of two observations.*

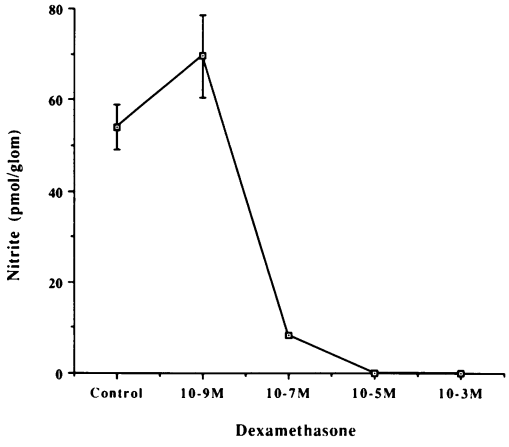

Figure 3. *Effect of dexamethasone on LPS stimulated glomerular nitrite production. Glomeruli were isolated 4 days after induction of glomerulonephiritis and incubated with LPS 1 μg/ml and various concentrations of dexamethasone (n = 3).*

lonephritis. Secondly it may be due to a systemic effect of the inflammation in the left kidney, producing activation of cells in the right. A third possibility is that it reflects part of the functional response of the right kidney to reduction in glomerular filtration in the left kidney.

There are four cell types: macrophages, neutrophils, endothelial cells, and mesangial cells, that are present in nephritic glomeruli that have previously been shown to produce NO. It is now clear that there are two distinct NO synthase enzymes.[20,21] The first of these is constitutively present in vascular endothelium, platelets, and brain.[22] The second enzyme is inducible in macrophages,[9,10] endothelial cells,[23] and mesangial cells[24] in response to stimuli such as cytokines, and once stimulated leads to the synthesis of larger quantities of NO for up to 72 hours

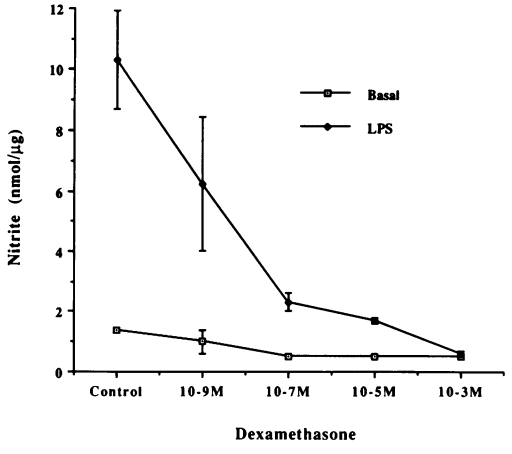

Figure 2. *Effect of dexamethasone on nitrite production by thioglycollate elicited peritoneal macrophages. Macrophages were incubated for 48 hours under basal conditions or with LPS 1 μg/ml. Dexamethasone was added 2 hours before LPS. Each point is mean ± sd of duplicate wells.*

in culture.[9] For macrophages, gamma interferon is the major stimulus of NO production,[10] whereas for mesangial cells, interleukin-1 (IL-1) and tumor necrosis factor (TNF) are effective.[24] The production of NO by nephritic glomeruli is similar to that seen with stimulated macrophages, in that it is produced in a linear fashion over 48 hours in culture, is increased by LPS stimulation and, as we have now shown, is inhibited by dexamethasone. We have also shown that macrophages isolated from nephritic glomeruli are able to produce NO_2^- and that production by these macrophages is approximately doubled by LPS stimulation, as is seen with whole nephritic glomeruli. We have found that macrophages isolated from glomeruli at day 4 after induction of nephritis make 4.9 nmols/10^5 cells. We have previously shown that there are a mean of 381 macrophages/glomerulus on day 4 of this model,[14] and so these cells would produce 18.7 pmol/glomerulus, which is not dissimilar to the level of 24.4 pmol we have measured on day 4. In contrast, bovine mesangial cells[24] were shown to make approximately 6.5 nmol/10^6 cells of nitrite and nitrate combined after TNF stimulation; even assuming this were all nitrite and that there are 250 mesangial cells/glomerulus, this would only account for 1.6 pmol/glomerulus. Taken together these facts suggest that macrophages are the major source of glomerular nitrite in our nephritic glomeruli. We cannot exclude the possibility, however, that glomerular cells or other intrinsic glomerular cells make some contribution to the total NO_2^- production, and this is especially true for the right control kidneys, where the levels are much lower and could certainly represent mesangial cell production.

It is of interest that the synthesis of NO reaches a peak at 4 days and then declines by 7 days, even though in previous experiments[14] we have shown that there is still a slight increase in macrophage numbers over this time. A

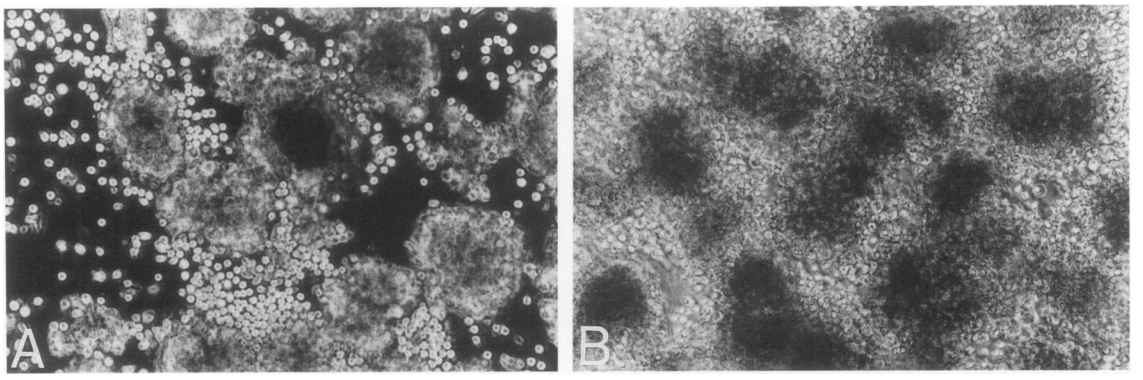

Figure 4. *Effect of dexamethasone on migration of macrophages from nephritic glomeruli. Glomeruli were isolated 4 days after induction of glomerulonephritis and incubated with dexamethasone $10^{-5}M$* **(A)** *or $10^{-9}M$* **(B)**. *Culture wells were photographed after 48 hours. With $10^{-9}M$* **(B)** *dexamethasone there is an almost confluent sheet of macrophages that have migrated from the glomeruli, whereas at the higher concentration of dexamethasone* **(A)** *macrophage migration is reduced.*

similar decline in NO synthesis was seen in nephrotoxic nephritis[13] after a peak at 24 hours, even though there was no change in numbers of glomerular macrophages. It may be that this reflects down-regulation of the NO synthase with time. Another possibility is that increased synthesis of arginase, which is known to occur with macrophage activation,[25,26] reduces the availability of L-arginine for the NO synthase pathway.

Glucocorticoids are known to have diverse effects on macrophages, including the suppression of their accumulation in infective foci,[27] inhibition of Fc and C_3 receptors,[28] reduction of proteinase[29] and IL-1 secretion,[30] and reduction of Ia expression.[30] We have now demonstrated that dexamethasone reduces NO synthesis both by peritoneal macrophages and by nephritic glomeruli. Holdsworth and Bellomo[31] have previously shown that dexamethasone ameliorates anti-glomerular basement membrane mediated glomerulonephritis in the rabbit, an effect that depended on inhibition of macrophages into glomeruli. Our results suggest a further mechanism by which glucocorticoids may affect macrophage-dependent glomerulonephritis. Also of interest is that, in addition to the inhibition of NO production, dexamethasone inhibited macrophage migration from nephritic glomeruli *in vitro* at doses that did not affect macrophage viability.

There are several effects that may result from NO production within nephritic glomeruli. Firstly NO is, at least in part, responsible for cytotoxicity of activated macrophages.[32] This effect is mediated through iron-nitrosyl-sulphur complex formation with inhibition of multiple iron-dependent enzymes and cessation of DNA replication. Thus NO may cause damage to intrinsic glomerular cells in acute glomerulonephritis. Secondly basal NO production in the kidney has been shown to modulate the glomerular filtration rate and filtration fraction,[33] possibly in part by its effect on inhibition of mesangial cell contraction,[34] and increased NO production in glomerulonephritis may have hemodynamic effects. Thirdly it has been shown that NO may inhibit mesangial cell mitogenesis[35] and so may modulate mesangial proliferation in glomerulonephritis.

In conclusion, we have now demonstrated NO production in a model of acute *in situ* immune complex glomerulonephritis in the rat. As with acute nephrotoxic nephritis,[13] we believe that infiltrating macrophages are the major source of NO. Nitric oxide production *in vitro* can be completely inhibited by glucocorticoids in this model. Further studies are necessary to determine the role of NO in glomerulonephritis.

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