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Lipopolysaccharide induces inducible nitric oxide synthase-dependent podocyte dysfunction via a hypoxia-inducible factor 1 α and cell division control protein 42 and Ras-related C3 botulinum toxin substrate 1 pathway

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Abstract

Urine protein loss in immune complex-mediated diseases such as lupus nephritis is associated with podocyte foot process effacement (podocytopathy) but is not always dependent on glomerular immune complex deposition. Several murine and human studies have associated lupus nephritis with inducible nitric oxide synthase (iNOS) expression in what appear to be podocytes. This study was conducted to determine mechanisms of immune-complex-independent and iNOS-dependent podocyte dysfunction. Conditionally immortalized podocytes were cultured with lipopolysaccharide (LPS) and nitric oxide (NO), superoxide (SO), or peroxynitrite donors in the presence or absence of inhibitors of iNOS, reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase or monocyte chemotactic protein 1 (MCP-1), or with sepiapterin to increase coupling of iNOS homodimers. Podocyte NO, SO, and MCP-1 production and nitrotyrosine modifications were determined. The podocytopathy phenotype was determined by measuring cell motility and membrane permeability to albumin. This study determined that NO produced by iNOS is sufficient and necessary to induce podocytopathy. NO probably induces this phenotype via hypoxia-inducible factor 1 α and cell division control protein 42 and Ras-related C3 botulinum toxin substrate 1 pathways. With LPS stimulation, neither SO nor peroxynitrite produced by uncoupled iNOS or NADPH oxidase nor MCP-1 was sufficient to induce the full phenotype. This study supports the notion that iNOS may induce autocrine podocyte dysfunction. Thus, targeting iNOS or the pathways of its induction may have therapeutic benefit.

Keywords

Podocyte; Toll-like receptor 4; Nitric oxide; Superoxide; Lipopolysaccharide; Hypoxia-inducible factor 1 α ; Cell division control protein 42; Ras-related C3 botulinum toxin substrate 1; Inducible nitric oxide synthase; Lupus nephritis; Peroxynitrite; Free radicals

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Introduction

Proteinuria is a cardinal sign of lupus nephritis (LN). The extent and the kinetics of proteinuria constitute a major independent predictor of prognosis and response to therapy [1]. In LN as well as other nephropathies, proteinuria plays a central role in progressive renal dysfunction [2]. The location of immune complex deposition in the subendothelial, mesangial, or subepithelial space has been thought to determine the type of glomerular injury leading to proteinuria [3,4]. Anti-DNA antibodies, prominent in many cases of LN, can lead to proteinuria through activation of the complement cascade, activation of Fc receptors, or translocation to the nucleus to activate Toll-like receptors (TLRs) [5]. However, nephrotic syndrome in patients with LN can occur without immune complex (IC) deposition or glomerular cellular infiltration/proliferation [4]. In addition, the association between glomerular immune complex deposits and the extent of proteinuria is not tight. Patients with nephrotic syndrome have more extensive podocyte foot process effacement than patients with nonnephrotic proteinuria, highlighting the role of podocyte dysfunction in nephrotic proteinuria [4]. It is logical to conclude that the podocyte injury in lupus patients with nephrotic syndrome may involve pathways other than or in addition to immune complex deposition. The factors that govern the variability in endocapillary and podocyte pheno-type, and hence the degree of proteinuria, are still unknown.

Several studies showed a strong link between inducible nitric oxide synthase (iNOS) expression and development and severity of the disease phenotype in several murine models of lupus nephritis [6,7]. Both MRL/MpJ-Fas^{lpr}/J (MRL/lpr) and (New Zealand Black × New Zealand White)_{F1} mice develop spontaneous proliferative lupus nephritis. MRL/lpr mice developed increasing levels of urine nitric oxide (NO^{*}) metabolites in parallel with or preceding the onset of glomerulonephritis [8]. Interventional studies utilizing competitive inhibitors of iNOS suggest that iNOS activity modulates proteinuria in murine lupus. Inhibiting NOS activity in MRL/lpr mice before disease onset with the nonspecific arginine analog L-N^G-monomethyl-L-arginine had a limited effect on cellular proliferation and necrosis within the glomerulus [8,9]. In addition, this effect occurred in the absence of a change in immunoglobulin or complement deposition in the glomerulus, suggesting that increased iNOS expression occurred downstream or independent of immune complex deposition and complement activation [8]. On the other hand, mice given the inhibitor did not develop proteinuria throughout the study [9], suggesting a protective role of NOS inhibition in preventing podocyte injury or dysfunction. This protective effect of NOS inhibition was also supported by a study utilizing N⁶-(1-iminoethyl)-L-lysine, dihydrochloride (L-NIL; a partial, selective inhibitor of iNOS), although the dose used was not selective for inhibition of iNOS [7]. In this study, proteinuria was absent in LNIL-treated MRL/lpr mice lacking the NOS2 gene. Podocytes also showed less foot process (FP) effacement, suggesting a protective effect of NOS inhibition in non-iNOS isoforms. Thus, NOS activity inhibition in the context of lupus nephritis is protective against proteinuria and podocyte FP effacement independent of IC and complement deposition. This suggests a role for NOS activity in podocyte dysfunction in LN.

In previous studies, podocytes expressed TLRs 1–6 and 9 [10]. However, only TLR-3 and TLR-4 agonists affected podocyte actin cytoskeleton and CD80 (B7-1) expression, a marker

of podocyte activation. TLR-3 signaling was NF- κ B dependent. However, no signaling pathways are known for TLR-4 agonist-mediated podocyte dysfunction. TLR activation by several endogenous ligands is important in multiple diseases of the vasculature, such as myocardial infarction and atherosclerosis [11,12]. Several TLR-4 ligands are endogenous, including heat shock proteins, fibronectin, hyaluronic acid, and fibrinogen [13,14]. These endogenous TLR-4 ligands can be detected in the setting of human progressive glomerular diseases. In murine membranoproliferative glomerulonephritis, endogenous TLR-4 ligands have been implicated in disease [15]. Also fibrinogen, an endogenous TLR-4 agonist, was shown to be deposited along podocytes in Bowman's space in NEP25 mice, a model of glomerular sclerosis (NEP25 mice) [16]. All of these studies suggest that endogenous agonists of TLR-4 are present in glomerular diseases that may induce podocyte dysfunction independent of TLR7/9 activation seen with endocytosed DNA and RNA immune complexes in systemic lupus erythematosus. Determining the mechanism of podocyte dysfunction could lead to targeted therapies for membranous nephropathy and nephrotic syndrome in lupus patients.

NO $\cdot$ stabilizes and activates hypoxia-inducible factor 1 α (HIF-1 α) under normoxia conditions, thus leading to conditions that emulate decreased oxygen availability. NO $\cdot$ may act by blocking prolyl hydroxylase activity [17], activating transcription/translation control pathways of HIF-1 α [18], or regulating target protein stability through S-nitrosylation [19]. Rac1 and CDC42 are known to induce podocyte foot process motility and process effacement, leading to proteinuria [20].

The goal of this study was to determine the role of NO $\cdot$ and iNOS-mediated superoxide (O $_2^{\bullet-}$) production in podocyte dysfunction and to determine the mechanism of reactive intermediate-mediated podocyte dysfunction. In this study, conditionally immortalized podocytes stimulated with these TLR agonists produced NO $\cdot$ and O $_2^{\bullet-}$ with an effect on podocyte phenotype. Moreover, this study demonstrated that NO $\cdot$ induced the motile phenotype and loss of protein barrier function in a HIF-1 α and small GTPase-dependent fashion.

Materials and methods

Conditionally immortalized podocytes

Because primary cultures of differentiated podocytes do not proliferate well and may be contaminated by other glomerular cell types, a conditionally immortalized podocyte cell line was used. This line was created using a transgenic mouse (H-2 K^b-tsA5) with an SV40 large T antigen that was temperature sensitive and controlled by an interferon- γ -inducible promoter antigen [21]. Cells that are grown under nonpermissive conditions (37 °C, no interferon- γ) are phenotypically equivalent to primary differentiated podocytes (i.e., they have an arborized appearance and express synaptopodin). Under permissive conditions (33 °C, interferon- γ) these cells grow in a cobblestone morphology with low expression of synaptopodin, not like primary podocytes [21].

Conditionally immortalized podocytes, kindly provided by Dr. Jeffery Kopp (National Institute of Diabetes, Digestive, and Kidney Diseases, Bethesda, MD, USA), were cultured

as previously described [22]. Podocytes were grown in collagen-I-coated (BD BioCoat, BD Biosciences, San Jose, CA, USA) culture plates at 33 °C with 20 U/ml mouse recombinant interferon- γ (Sigma Chemical Co., St. Louis, MO, USA) to enhance expression of a thermosensitive T antigen until 90% confluence was reached (6–7 days). Under these conditions, cells proliferated and were undifferentiated. To induce differentiation and reduce proliferation, podocytes were grown at 37 °C in the absence of interferon- γ for 8 days. Podocyte maturity was examined by assessing cell morphology and synaptopodin expression (data not shown). RPMI 1640+GlutaMAX (Invitrogen, Grand Island, NY, USA) with 10% fetal calf serum (FCS; Invitrogen), penicillin (100 units/ml), and streptomycin (Invitrogen, 100 μ g/ml) was used as culture medium. Cells were incubated with 1% FCS RPMI for 24 h before each experiment. The cells, at 1.5×10^4 and 1.5×10^5 cells/ml, were allowed to grow under permissive and nonpermissive conditions, respectively, on 35-mm tissue culture plates coated with type I collagen (BD Biosciences). Podocyte morphology was acquired using a Nikon Digital Sight DS-SM camera mounted on a Nikon Eclipse TS100 microscope (Nikon Instruments, Melville, NY, USA). To further confirm the phenotype of the cells to be used in experiments, cells were grown under permissive and nonpermissive conditions on collagen type I-coated glass coverslips for immunocytochemistry and fixed using 2% paraformaldehyde, 4% sucrose in phosphate-buffered saline (PBS) for 5 min (at room temperature). Then the cells were permeabilized with 0.3% Triton X-100 (in PBS) for 5 min at room temperature. The cells were rinsed with PBS, and then nonspecific binding sites were blocked in blocking solution (0.1% Tween 20 (Sigma) with 4% fetal calf serum in PBS) for at least 30 min. Primary monoclonal anti-synaptopodin (Fitzgerald, Acton, MA, USA) and FITC-conjugated anti-mouse IgG1 (Jackson ImmunoResearch, Philadelphia, PA, USA) were used, and the coverslips were mounted with 15% Mowiol (Calbiochem, La Jolla, CA, USA). Specimens were analyzed using a Nikon Eclipse 80i microscope and camera (DS-Qi1Mc, Nikon Instruments).

Scratch-wound assay

Differentiated podocytes were grown as monolayers in 35-mm tissue culture plates coated with type I collagen (BD Biosciences). The cells were scratched with a sterile 200- μ l pipette tip to create a cell-free area. Afterward, the culture medium was refreshed and either lipopolysaccharide (LPS; 100 ng/ml, Sigma) or diethylenetriamine (DETA) NONOate (100 μ M, Enzo Life Sciences, Farmingdale, NY, USA) was added. Pictures were taken under 10 $\times$ magnification at 0 and 12 h after the scratching procedure. The number of cells that had migrated into a standardized field was counted.

Lucigenin assay

Differentiated podocytes (2×10^5 cells per well) were plated in a collagen-I-coated 12-well plate (BD Biosciences). The cells were then given various treatments (LPS, 100 ng/ml; poly(I:C), 300 ng/ml; arginine, 1 mM) for 18 h. The cells were homogenized in buffer (pH 7.4) with 150 mM NaCl, 50 mM Tris, 25 mM ethylene glycol tetraacetic acid, and 25 mM ethylenediaminetetraacetic acid with a protease inhibitor and phosphatase inhibitor cocktail (source). Freshly prepared homogenates (100 ml) were incubated for 30 min at 37 °C with no treatment, or with ascorbate as a negative control, or with one of the following inhibitors of known $O_2^{\bullet-}$ -diphenylene -producing enzymes: diphenylene iodonium (50 mM) or 7-(1,3-

benzoxazol-2-ylsulfanyl)-3-benzyl-3*H*-[1-3]triazolo[4,5]-*d* pyrimidine (VAS2870; 10 μ M, Life Sciences). Incubated samples (50 μ l) were placed in a 24-well reading plate containing 25 μ M lucigenin (Sigma) for the detection of $O_2^{\bullet -}$ in 500 μ l final volume of Krebs solution with 10 mM Hepes–NaOH (pH 7.4). Readings for each sample were performed using a Luminoskan luminometer (Thermo Scientific, Waltham, MA, USA) at 37 °C in triplicates and normalized to the protein level measured by protein assay (Bio-Rad, Hercules, CA, USA) after background subtraction.

Nitrate and nitrite assay

Differentiated podocytes (12×10^5 cells per well) were plated in a collagen-I-coated 12-well plate (BD Biosciences). Cells were then given various treatments (LPS, 100 ng/ml; poly(I:C), 300 ng/ml; arginine, 1 mM) for 18 h in phenol-free, nitrate/nitrite-free RPMI at 37 °C. The supernatant was collected and filtered. Readings for each sample (100 μ l) were performed using a chemiluminescence detector (Model 280i; Sievers, Boulder, CO, USA), with vanadium chloride at 95 °C as a reducing agent as described [23]. Measurements were performed in triplicates and reported in micromolar concentrations.

3-Nitrotyrosine competitive ELISA

Podocytes (2×10^5 per well) were treated for 18–20 h. SIN-1 (50 μ M) was added as a positive control. Cells were lysed and analyzed for 3-nitrotyrosine (3NT) by competitive ELISA (Oxiselect, Cell Biolabs, San Diego, CA, USA) per the manufacturer's instructions.

Cytokine measurement

Monocyte chemotactic protein 1 (MCP-1) was measured in triplicate in the supernatant of cultured podocytes (2×10^5 cells per well) using an ELISA kit (eBioscience, San Diego, CA, USA), using the manufacturer's instructions and reported in pg/ml concentrations.

Transwell permeability assay

This assay was performed following the previously published protocol [24] with some modifications. Differentiated podocytes (2×10^5 cells per well) were grown as a monolayer on 3.0- μ m-pore collagen-I-coated transwells (Corning, Lowell, MA, USA) in a 12-well tissue culture plate. The confluence was checked by the measurement of transepithelial resistance (Millicell-ERS by Milli-pore, Danvers, MA, USA) that averaged $105.77 \pm 31.4 \Omega \text{ cm}^2$ around day 7 under nonpermissive conditions. Cells were pretreated as below in 0.5% RPMI for 4 h before stimulation with 100 ng/ml LPS, 300 ng/ml poly(I:C), or 1 mM arginine for another 4 h. the cells were washed twice in PBS with 1 mM each MgCl_2 and CaCl_2 . The upper compartment was refilled with 0.25 ml RPMI alone, and the lower compartment was filled with 0.5 ml RPMI 1640 supplemented with 60 mg/ml bovine serum albumin and incubated for 2 h at 37 °C. Upper chamber protein concentration was measured using a Bio-Rad protein assay (Bio-Rad Laboratories).

To determine if the podocyte conditions induced nonspecific permeability, dextran permeability assays were performed using FITC–dextran at 40 and 150 kDa (Molecular Probes, Eugene, OR, USA). These sizes were chosen because they were above and below that of albumin. The same procedure described above was followed for the dextran

experiments. Dextrans were applied to the upper chamber (0.25 mg/ml) after washing with PBS. Fluorescence activity of the medium in the lower chambers as a reflection of the podocyte monolayer permeability was measured by a fluorescence plate reader (excitation 495 nm, emission 520 nm; Molecular Devices, Sunnyvale, CA, USA) after 90 min.

Lactate dehydrogenase (LDH) cytotoxicity assay

To determine if cell death was a cause of loss of cell barrier function in these studies, LDH was measured in the supernatant of cultured podocytes treated with LPS (100 ng/ml), poly(I:C) (300 ng/ml), and arginine (1 mM) using the Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific), following the manufacturer's instructions. Measurements were performed in triplicate.

Statistical methods

Comparisons between groups were made using Student's t test if the data were normally distributed or Wilcoxon rank sum if not. Data are displayed as means $\pm$ standard error. Differences between groups were deemed significant if p values <0.05 were observed.

Results

Conditionally immortalized podocytes showed classical synaptopodin expression and arborized morphology under nonpermissive (differentiating) conditions

To confirm that the conditionally immortalized podocytes used are structurally and functionally mature at the time of the experiments, the expression of synaptopodin at day 7 was determined under permissive conditions (33 °C, interferon- γ) and at day 7 under nonpermissive conditions (37 °C, no interferon- γ) using fluorescence microscopy. Synaptopodin was strongly expressed in the cytoplasm of cells grown under nonpermissive conditions, indicating differentiation. Examination of cell morphology using light microscopy showed clear arborizations that correspond to foot process formation under nonpermissive conditions.

Both NO $^{\bullet}$ and O $_2^{\bullet-}$ production increases upon stimulation of podocytes with TLR-4 agonist (LPS)

Lupus nephritis glomeruli develop a condition of chronic inflammation due to the uncontrolled activation of both the adaptive and the innate immunity. To induce an inflammatory milieu similar to lupus nephritis glomerulus, cultured podocytes were exposed to various TLR agonists. Upon stimulation, podocyte response to inflammation was measured by assessing the production of NO $^{\bullet}$, O $_2^{\bullet-}$, and cytokines. Cells stimulated with TLR-4 agonist (LPS) demonstrated a significant increase in NO $^{\bullet}$ production (Fig. 1A). Cells stimulated with both LPS and TLR-3 agonist (poly(I:C)) showed significant increases in O $_2^{\bullet-}$ production (Fig. 1B). Cells treated with L-NIL (10 μ M), a partially selective iNOS inhibitor, showed a reduced increase in NO $^{\bullet}$ production with LPS stimulation (Fig. 1A). In addition, treatment with L-NIL reduced O $_2^{\bullet-}$ production in LPS-stimulated cells, suggesting that uncoupled NOS contributed to O $_2^{\bullet-}$ production (Fig. 1B). As expected, arginine increased NO $^{\bullet}$ production but it had no significant effect on O $_2^{\bullet-}$ production (Fig. 1A and B).

Therefore, podocytes stimulated with LPS increased $\text{NO}^\bullet$ and $\text{O}_2^{\bullet-}$ production; this increase in production was reduced with NOS inhibition using L-NIL.

Podocyte MCP-1 production is increased upon stimulation with LPS in an $\text{NO}^\bullet$ -independent manner

Cytokines play an important role in the propagation and amplification of inflammation in the lupus nephritis glomerulus [25]. Podocytes, resident cells in the glomerulus, could be a major source of cytokines that lead to podocytopathy. Stimulation of podocytes with LPS increased MCP-1 production. Treatment with L-NIL had no effect on MCP-1 production (Fig. 2). This suggests that the MCP-1 production by podocytes upon LPS stimulation is $\text{NO}^\bullet$ independent.

Podocyte motility is increased upon LPS stimulation or addition of $\text{NO}^\bullet$

As discussed before, the motile phenotype of podocytes in vitro corresponds to foot process effacement in vivo. LPS increases both $\text{NO}^\bullet$ and $\text{O}_2^{\bullet-}$ production in stimulated podocytes (Fig. 1). To test the effect of LPS on podocyte motility, a scratch-wound assay was used. Both LPS and $\text{NO}^\bullet$ increased migration of podocytes (Fig. 3). $\text{O}_2^{\bullet-}$ had no effect (data not shown). This suggests that LPS and $\text{NO}^\bullet$ enhance the motile phenotype in cultured podocytes.

Stimulation with LPS or NO increases podocyte permeability to albumin

Cultured podocytes form unique cell–cell junctions that exhibit charge- and size-selective permeability [26]. The transwell permeability assay is a standard technique employed to test paracellular permeability across a monolayer of podocytes [26–29]. To validate the integrity and the reproducibility of the method, two factors must be considered: (1) the integrity of the podocyte monolayer and (2) the cytotoxicity of the treatment of podocytes. The confluence of the podocyte monolayer was ensured to be consistent through experiments by measuring transepithelial resistance, which averaged $105.7 \pm 31.4 \, \Omega \, \text{cm}^2$ at day 7 under nonpermissive conditions. Podocytes stimulated with LPS (100 ng/ml), poly(I:C) (300 ng/ml), and arginine (1 mM) for 4 h exhibited no increase in LDH activity, indicating no cytotoxic effect of the treatment used at a given dose and duration (data not shown).

To confirm a size selectivity of the permeability model, dextran permeability assays were performed using FITC–dextran at 40 kDa (comparable to the size of albumin) and 150 kDa (larger than albumin) [26] (Fig. 4A). As expected, in the absence of any cellular barrier, permeability is highest, with no significant difference between 40- and 150-kDa FITC–dextran permeability. Podocytes receiving no treatment had the lowest permeability for both 40- and 150-kDa dextran. Treatment with LPS or DETA ($\text{NO}^\bullet$ donor) significantly increased permeability for 40-kDa dextran but had no effect on the permeability of the 150-kDa dextran. This suggests that the treatments have no effect on monolayer integrity but rather on monolayer barrier function. This is confirmed by significantly reducing LPS-induced 40-kDa permeability with an NOS inhibitor (L-NIL).

After establishing the experimental model of the transwell assay, we tested the effect of podocyte stimulation on permeability using more physiologically relevant albumin (Fig.

4B). Cells were stimulated with LPS, poly(I:C), and arginine. Only LPS increased podocyte permeability. This increase was significantly inhibited in cells treated with L-NIL. This suggests that the LPS-induced increase in podocyte permeability is mediated by NOS, probably iNOS. NO is the principle product of NOS under normal conditions. To test the potential effect of NO[•], podocytes were treated with an NO[•] donor (DETA NONOate, 100 μM, which released similar levels of NO[•] compared to LPS stimulation). Addition of an NO[•] donor increased the permeability of podocytes for dextran 40 kDa (Fig. 4A). This suggests that NO[•] plays an important role in mediating LPS-induced permeability in cultured podocytes.

Exclusion of alternative mechanisms for LPS-induced loss of podocyte barrier function

Thus far, the experiments show that podocytes produce both NO[•] and O₂^{•-} upon activation with LPS. In addition, LPS induced the motile phenotype in these cells as demonstrated by the increased motility and increased paracellular permeability for dextran 40 kDa and albumin. NO[•] synthase and NO[•] seem to play an important role in conveying the LPS-induced signal. However, LPS concomitantly induces MCP-1 production in stimulated podocytes. MCP-1 was shown to enhance the motile phenotype and increased podocyte permeability to albumin in a transwell assay by acting in an autocrine fashion via the cysteine–cysteine chemokine receptor 2 (CCR2) [27]. The diagram in Fig. 5 shows possible mediators of the LPS signal in cultured podocytes. To decipher the most important pathway(s) involved in the LPS-mediated motile phenotype, podocyte permeability was assessed after treatment with various inhibitors/donors as specified in the diagram. L-NIL (10 μM), a partially selective iNOS inhibitor, and 1400 W (GW100) dihydrochloride (*N*-([3-(aminomethyl)phenyl]methyl)ethanimidamide dihydrochloride; 50 μM), a potent, selective iNOS inhibitor [30], were used to inhibit NOS production of NO[•] and potentially O₂^{•-} when uncoupled. DETA NONOate (100 μM) [31], a long-acting NO[•] donor, was used to add NO[•] to the culture conditions. BH₄ (tetrahydrobiopterin; 100 μM) was used as a coupling agent to enhance NOS-mediated NO[•] production and to reduce NOS-mediated O₂^{•-} production [32]. VAS2870 (10 μM) [33], a selective NADPH oxidase (NOX) inhibitor, was used to inhibit O₂^{•-} produced by NOXs. A xanthine (X) /xanthine oxidase (XO) system [34] (X 100 μM/XO 5 mU/ml) was used to generate O₂^{•-}. Anti-MCP-1 neutralizing antibody (5 μg/ml ab25124, Abcam, Cambridge, MA, USA) was used to neutralize MCP-1 binding to the CCR2 receptor in podocytes.

LPS-induced podocyte barrier permeability is not MCP-1 dependent

MCP-1 was shown to enhance the motile phenotype and increased podocyte permeability to albumin in a transwell assay by acting in an autocrine manner via CCR2 [27]. In the experiments, LPS induced MCP-1 production by stimulated podocytes. This raises the possibility that LPS can induce a motile phenotype in podocytes by increasing MCP-1 levels in an autocrine/paracrine fashion. To address this hypothesis, anti-MCP-1 neutralizing antibody was used along with LPS stimulation (Fig. 6). Note that the experiments have shown that LPS stimulation increases MCP-1 levels to 1.5 ng/ml per 2 × 10⁵ cells (Fig. 2). However, the lowest concentration at which addition of MCP-1 had an effect comparable to LPS was 50 ng/ml. The addition of MCP-1 along with LPS stimulation had no additive

effect on podocyte permeability. There was no significant difference between cells stimulated with LPS alone and cells stimulated with LPS in the presence of anti-MCP-1 neutralizing antibody. This suggests that the LPS-induced increase in podocyte permeability is not MCP-1 dependent. The MCP-1 effect was significantly reduced using the neutralizing antibody but not a rabbit preimmune IgG (Abcam) control.

LPS-induced podocyte barrier permeability is not NOX dependent in podocytes

The experiments have shown that LPS increases both $\text{NO}^\bullet$ and $\text{O}_2^{\bullet-}$ production in stimulated podocytes (Fig. 1). Treatment with L-NIL decreased LPS-induced permeability (Fig. 4). $\text{O}_2^{\bullet-}$ has many enzymatic and mitochondrial sources. Screening for possible sources using different inhibitors has shown that NOX and NOS contribute significantly to $\text{O}_2^{\bullet-}$ production in podocytes stimulated with LPS (data not shown). To test the role of NOX $\text{O}_2^{\bullet-}$ in LPS-induced permeability in podocytes, cells were stimulated in the presence of a specific NOX inhibitor (VAS2870 10 μM), to prevent NOX-mediated $\text{O}_2^{\bullet-}$ production, and xanthine with xanthine oxidase as a positive control. The outcomes measured include transwell permeability and $\text{O}_2^{\bullet-}$ and $\text{NO}^\bullet$ production. Treatment with VAS2870 had no effect on permeability in stimulated cells, in contrast to results on L-NIL permeability (Fig. 7A). This suggests that NOX-derived superoxide is not contributing to the permeability phenotype. Stimulated cells treated with combined L-NIL and VAS2870 had a permeability that was no different from those treated with L-NIL alone. This suggests that NOS is essential in mediating LPS-induced permeability. Addition of $\text{O}_2^{\bullet-}$ (X/XO) had no effect on podocyte permeability, further supporting the notion that superoxide does not signal for this phenotype and that NOS-induced podocytopathy is not dependent on $\text{O}_2^{\bullet-}$ production.

Both VAS2870 and L-NIL significantly reduced $\text{O}_2^{\bullet-}$ production in stimulated podocytes (Fig. 7B). This suggests that both NOX and uncoupled NOS contribute to $\text{O}_2^{\bullet-}$ production. As expected VAS2870 had no effect on $\text{NO}^\bullet$ production, unlike L-NIL, which significantly reduced $\text{NO}^\bullet$ production by stimulated podocytes (Fig. 7C).

Restoring NOS dimerization does not improve LPS-induced podocyte barrier function

All of the previous experiments indicate that $\text{NO}^\bullet$ synthase is essential in mediating LPS-induced podocyte permeability (Fig. 4). In addition, the reduction in $\text{O}_2^{\bullet-}$ production with an NOS inhibitor (L-NIL) suggests that uncoupled NOS is a significant source of $\text{O}_2^{\bullet-}$ (Fig. 7B). To test the relative contributions of NOS $\text{NO}^\bullet$ and NOS $\text{O}_2^{\bullet-}$ to LPS-induced podocyte permeability, BH_4 was used as an NOS coupling agent. In all three isoforms of NOS, BH_4 is a cofactor involved in the reduction of NOS heme iron to form an iron-oxy species that hydroxylates L-arginine to produce $\text{NO}^\bullet$. In the absence of BH_4 , the NOS enzymes produce $\text{O}_2^{\bullet-}$ rather than $\text{NO}^\bullet$, a situation referred to as NOS uncoupling [32]. In this experiment, stimulated podocytes were treated with BH_4 (100 μM) before transwell permeability and $\text{O}_2^{\bullet-}$ and $\text{NO}^\bullet$ production were measured (Fig. 8). Addition of BH_4 had no effect on LPS-induced permeability (Fig. 8A). In addition, stimulated podocytes treated with BH_4 showed decreased $\text{O}_2^{\bullet-}$ production (Fig. 8B) but no significant difference in $\text{NO}^\bullet$ production (Fig. 8C). This suggests that NOS induces the motile phenotype in podocytes through the

production of NO[•]. This observation is confirmed by the experiments employing the addition of NO[•] (DETA) without LPS stimulation (Fig. 4).

The LPS-induced leaky podocyte phenotype does not associate with ONOO⁻

Because the protein barrier function of podocytes is improved by NOS inhibition, it is possible that ONOO⁻ formed by NOS-derived NO and O₂^{•-} is also a pathogenic mediator. To explore this possibility, cells were treated with LPS and then cultured with NOS and NOX inhibitors as well as an NO donor, as in Fig. 7, but with the addition of a ONOO⁻ donor, SIN-1. Cell lysates were analyzed for 3NT protein modifications (indicative of ONOO⁻ production). As a demonstration of the assay, addition of SIN-1, a ONOO⁻ donor, to untreated cells resulted in increased lysate 3NT levels, whereas addition of DETA NONOate or X/XO did not (Fig. 9A). As expected, LPS treatment resulted in 3NT modifications, and these modifications were reduced with NOS inhibitors (GW100/W1400 or L-NIL) but not NOX inhibitors (VAS2870 or VAS). Suggesting that uncoupled NOS was a source of 3NT in this setting, sepiapterin (a BH₄ precursor that couples NOS) reduced 3NT modifications to cell proteins induced by LPS (Fig. 9B). Whereas the leaky podocyte phenotype was induced by addition of an NO donor (Fig. 4), the ONOO⁻ donor (SIN-1) did not induce the phenotype as well as LPS treatment, but did increase 3NT levels to equivalent to those seen with LPS treatment (Fig. 9B and C). Addition of the ONOO⁻ scavenger, melatonin [35,36], also failed to restore a normal protein barrier in LPS-treated cells while reducing 3NT levels. Addition of the NOS coupling agent, sepiapterin, reduced 3NT levels to untreated levels but did not reduce the leaky phenotype (Fig. 9B and C). These results combined suggest that ONOO⁻ is not the dominant reactive species mediating the observed phenotype induced by LPS.

Endogenous and exogenous NO[•] acts in a HIF-1 α and CDC42/Rac1-dependent fashion to induce podocyte barrier permeability

NO[•] is well known to stabilize HIF-1 α and activate HIF-1 α under normoxic conditions. In addition, HIF-1 α was shown to increase the levels of CDC42/Rac1, promoting motility. Thus, we hypothesized that LPS-induced NOS production of NO[•] promotes a motile phenotype in podocytes in a HIF-1/CDC42/Rac1-dependent pathway. To test our hypothesis, we used an iNOS-specific inhibitor (GW100/1400 W, 50 μ M), a CDC42/Rac1 inhibitor (ML141, 10 μ M; Calbiochem), and a HIF inhibitor (chetomin, 0.15 μ M; Enzo Life Sciences). In addition, DETA NONOate (100 μ M) was used as an exogenous NO[•] donor positive control. Podocytes were either exposed to NO[•] (Fig. 10A) or stimulated with LPS (Fig. 10B) in the presence of the above treatments, and albumin permeability was measured using a transwell assay. The CDC42/Rac1 and HIF inhibitors independently reduced both NO[•]-mediated (Fig. 10A) and LPS-induced (Fig. 10B) podocyte permeability. The iNOS inhibitor had no significant effect on unstimulated or NO[•] donor-induced podocyte permeability but significantly reduced LPS-induced podocyte permeability (Fig. 8B). This strongly suggests that iNOS is important for LPS-induced NO[•] production in podocytes. In addition, this study suggests that both HIF and CDC42/Rac1 are downstream of NO[•] production in mediating podocyte permeability.

Discussion

This study suggests a role for NO[•] in the podocyte motility and permeability phenotype in the presence of the TLR agonist LPS. In these studies, a TLR4 agonist increased markers of podocyte production of NOS-mediated NO[•], uncoupled-NOS-mediated O₂^{•-}, and NOX-mediated O₂^{•-}. The increased production of NO[•] was sufficient and necessary to enhance the motile phenotype and paracellular permeability of cultured podocytes. In addition, NO[•] and NOS activity were shown to act in a HIF-1 α and CDC42/Rac1-dependent manner. This is the first report that supports a link between increased NO[•] production and podocyte pathophysiology at the cellular level.

Until recently, podocytes were thought to be paracrine recipients of inflammatory mediators that lead to injury. Indeed, podocytes are highly susceptible to a number of pathogenic factors such as complement, reactive oxygen species (ROS), and toxins such as puromycin aminonucleoside. Podocytes upregulate their expression of C5a receptors [37] and are thus susceptible to complement-mediated immune injury in membranous nephropathy [38]. Against traditional models, recent studies support the notion that podocytes may contribute to feed-forward acceleration of glomerular injury through the expression of receptors linked to proinflammatory pathways [15].

Recent studies indicate that chemokines are expressed in podocytes after exposure to LPS [15], supporting the notion that they are active participants in inflammatory responses. How TLR-4 signaling is important physiologically in podocytes is not known, but its expression could give podocytes an early response role in clearance of bacterial infection, as LPS-induced permeability is potentially increased in patients with gram-negative sepsis. A prospective study showed that in postoperative patients, microalbuminuria indicates increased glomerular permeability, a finding that is unique to septic patients [39]. This proteinuria may be a physiological response to clear pathogenic molecules. Of interest, in those with minimal change disease, proteinuria often follows infection or an allergic episode [40]. Therefore, the sustained activation of TLR-4 by an endogenous agonist may contribute to proteinuria observed in membranous lupus nephritis. Recent studies suggest that endogenous ligands of TLR released during cellular necrosis signal for sterile inflammation and injury [41]. In addition, overexpression of TLR-4 in mice also resulted in autoantibodies and lupus-like nephritis in C57BL/6 mice [42].

These studies demonstrated that LPS-activated podocytes produce O₂^{•-}. The major enzymatic sources were shown to be uncoupled NOS and NOX. The Heymann nephritis model of membranous nephropathy is dependent on the formation of the C5b-9 membrane attack complex to induce proteinuria, possibly through upregulation of expression of the NADPH oxidase enzyme complex that is translocated to podocyte cell surfaces.

Subsequently, O₂^{•-} is produced locally and reaches the GBM matrix. O₂^{•-} initiates lipid peroxidation and subsequent degradation of GBM collagen IV, leading to proteinuria [43]. These injurious effects of O₂^{•-} were not assessed in the experiments owing to the relatively short exposure time. It is not yet clear if targeting ROS-producing enzymes will reduce podocytopathy in humans.

The current study links NOS to $O_2^{\bullet-}$ production in podocytes for the first time. In studies of endothelial (e) NOS-overexpressing transgenic mice with and without transgenic overexpression of BH_4 , the ratio of eNOS to BH_4 is important in the amount of $NO^{\bullet}$ produced relative to $O_2^{\bullet-}$ [44]. This suggests that eNOS is uncoupled in this setting, and evidence suggests similar uncoupling of iNOS can occur in lupus nephritis [45]. It is not yet clear from where the ROS that oxidize BH_4 originate; however, both iNOS and NADPH oxidase are important candidates in lupus nephritis [46]. NOS $O_2^{\bullet-}$, NOX $O_2^{\bullet-}$, and exogenous $O_2^{\bullet-}$ all had no immediate effect on podocyte permeability in these studies, suggesting that $O_2^{\bullet-}$ itself is not a mediator of the podocyte phenotype described in this study.

MCP-1 production increases upon podocyte stimulation with LPS. MCP-1 was shown to increase podocyte permeability upon stimulation with TGF- β [27]. However, in those experiments, supraphysiological levels of MCP-1 (50 ng/ml) were needed to increase podocyte permeability. In addition, we showed that the use of MCP-1-neutralizing antibodies did not affect LPS-induced permeability. In addition, inhibition of NOS did not affect MCP-1 production. This would suggest that MCP-1 is not involved in LPS- or $NO^{\bullet}$ -induced podocyte permeability.

The effect of $NO^{\bullet}$ on cell motility is cell and dose specific [47,48]. $NO^{\bullet}$ stabilizes HIF-1 α and activates HIF-1 under normoxia, thus eliciting functional responses that mimic hypoxia. Several mechanisms are implicated: $NO^{\bullet}$ may block prolyl hydroxylase activity [17], activate transcription/translation control pathways of HIF-1 α [18], or regulate its stability through S-nitrosylation [19]. In addition HIF-1 α stabilized by $NO^{\bullet}$ was shown to regulate small GTPase (CDC42/Rac1) transcription and promote cell migration [49]. Rac1 and CDC42 promote podocyte foot process motility and the development of foot process effacement and proteinuria [20].

The study has several limitations. First, the role of $ONOO^-$ in podocyte pathology was not addressed directly. However, reducing NOS-mediated $O_2^{\bullet-}$ production (and presumably NOS uncoupling) with BH_4 and sepiapterin did not restore the normal podocyte phenotype, suggesting that uncoupled NOS-derived $O_2^{\bullet-}$ or $ONOO^-$ was not important in podocyte dysfunction. Addition of a $ONOO^-$ donor also failed to induce the phenotype, and addition of a scavenger of $ONOO^-$ did not restore normal protein barrier function. This contrasts with sepsis studies demonstrating 3NT formation in LPS and sepsis-induced kidney injury and improved renal function with a $ONOO^-$ decomposition catalyst [50,51]. However, these studies were not designed to evaluate podocyte function. Inhibition of NADPH oxidase did not affect the leaky podocyte phenotype or change 3NT formation, suggesting that $O_2^{\bullet-}$ substrate from this enzyme does not contribute to $ONOO^-$ production in a fashion that affects phenotype or 3NT formation. In addition, the conclusions are based on changes in phenotype and markers of $NO^{\bullet}$, $O_2^{\bullet-}$, and $ONOO^-$ production and podocyte phenotype in add-back and pharmacologic inhibitor studies. Indirect measures of $NO^{\bullet}$, $ONOO^-$, and $O_2^{\bullet-}$ were used, making it impossible to be certain of the involvement of $NO^{\bullet}$ and $O_2^{\bullet-}$ versus

ONOO⁻ in the podocyte phenotype observed. However, the addition of appropriate inhibitor controls increases the strength of the conclusions.

This study suggests a critical role for iNOS/NO^{*} in LPS-induced podocytopathy. Because podocytes are a likely source of iNOS expression in lupus nephritis [52,53], this provides a mechanism independent of immune-complex-mediated complement activation or Fc receptor activation for podocytopathy in this disease. Because TLR-4 may be important in the progression of lupus-like nephritis in animal models, and endogenous TLR-4 ligands can be measured in lupus patients [54], it is logical to hypothesize that it may also be important in human disease [55]. This pathway may be important in other diseases such as diabetic nephropathy and IgA nephropathy in which TLR-4 may play a role [56,57]. Thus, targeting this pathway in membranous lupus nephritis may have therapeutic potential.

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Fig. 1.

Nitric oxide and superoxide production increases upon stimulation with LPS or poly(I:C) in cultured podocytes. Differentiated podocytes were stimulated for 18 h with Toll-like receptor agonists (LPS, poly(I:C)). (A) LPS stimulation significantly increased NO[•] production (supernatant NO₂/NO₃). Treatment with L-NIL inhibited LPS-stimulated NO[•] production. Arginine, as expected, increased NO[•] production. NT, no treatment. **p* < 0.05 compared to NT without L-NIL. ***p* < 0.05 compared to LPS without L-NIL (*n* = 4). (B) Both LPS and poly(I:C) stimulation significantly increased superoxide production. Treatment with L-NIL inhibited superoxide production in LPS-stimulated cells. NT, no treatment. **p* < 0.05 compared to no NT without L-NIL. ***p* < 0.05 compared to LPS without L-NIL (*n* = 4).

Fig. 2.

MCP-1 production increased upon stimulation of cultured podocytes with LPS.

Differentiated podocytes were stimulated for 18 h with LPS. MCP-1 production was significantly increased upon stimulation. Treatment with L-NIL had no effect on MCP-1 levels. NT, no treatment; IL-6, interleukin 6. * $p < 0.05$ compared to NT without L-NIL ($n = 3$).

Fig. 3.

Stimulation with LPS or addition of nitric oxide increased motility of cultured podocytes. Differentiated podocyte monolayers were scratched with a sterile pipette tip to create a cell-free, denuded area. After 12 h of culture, the number of cells in the denuded area was counted. Stimulation with LPS or addition of DETA NONOate (NO donor) significantly increased the number of migratory podocytes compared to no treatment (no treatment, 5 ± 7 cells; LPS, 46 ± 12 cells; NO, 38 ± 9 cells). $p < 0.05$ for both treatments ($n = 3$).

Fig. 4.

Podocyte monolayer exhibits size-selective dextran and albumin permeability with NO and LPS stimulation. Permeability was measured by determining the transwell fluorescence of different-sized fluorescent dextran beads after culture with podocytes or no barrier (no podocytes). (A) Podocytes receiving no treatment had the lowest permeability for both 40- and 150-kDa dextran. Treatment with LPS and DETA (NO donor) had no effect on 150-kDa dextran permeability yet significantly increased 40-kDa dextran permeability. Podocytes stimulated with LPS in the presence of L-NIL (10 μ M) had reduced permeability compared to podocytes receiving LPS alone. * p < 0.05 compared to no treatment 40-kDa dextran. ** p < 0.05 compared to LPS 40 kDa (n = 4). (B) Cells were stimulated with LPS (100 ng/ml), poly(I:C) (300 ng/ml), and arginine (1 mM) for 4 h. Only LPS increased podocyte permeability. This increase was significantly inhibited in cells treated with L-NIL (10 μ M). Podocytes treated with NO donor (DETA NONOate 100 μ M) showed increased permeability independent of L-NIL. NT, no treatment. * p < 0.05 compared to NT no L-NIL. ** p < 0.05 compared to LPS no L-NIL (n = 5).

Fig. 5.

A diagram representing the possible pathways through which LPS conveys its signal to induce the motile phenotype in cultured podocytes. LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; NOS, nitric oxide synthase; NOX, NADPH oxidase; MCP1, monocyte chemoattractant protein 1; NO[•], nitric oxide; O₂^{•-}, superoxide; L-NIL, N⁶-(1-iminoethyl)-L-lysine dihydrochloride; 1400 W (iNOS specific inhibitor), N-([3-(aminomethyl)phenyl]methyl)ethanimidamide dihydrochloride; DETA, diethylenetriamine NONOate; BH₄, tetrahydrobiopterin; VAS2870, 7-(1,3-benzoxazol-2-ylsulfanyl)-3-benzyl-3H-[1-3]triazolo[4,5]-d pyrimidine; X/XO, xanthine/xanthine oxidase. Blue arrows, donors. Red bars, inhibitors.

Fig. 6.

LPS-induced permeability is not MCP-1 dependent. Cells were stimulated with LPS (100 ng/ml) or treated with MCP-1 (50 ng/ml) for 4 h. LPS and MCP-1 increased podocyte permeability to albumin. The addition of anti-MCP-1 neutralizing antibody (5 μ g/ml mAb) had no effect on LPS-induced permeability but decreased the MCP-1 effect on podocyte permeability. IgG was added as a control. * p < 0.05 compared to no treatment. ** p < 0.05 compared to MCP-1 (n = 5).

Fig. 7.

LPS-induced permeability is less NOX or superoxide dependent in podocytes. Cells were stimulated with LPS (100 ng/ml) in the presence of L-NIL and/or VAS2870 (VAS) or xanthine + xanthine oxidase (X/XO) for 4 and 18 h. Albumin permeability was measured using the transwell assay. (A) Stimulated cells treated with VAS had no change in LPS-induced permeability, whereas X/XO-generated superoxide in unstimulated cells had no effect on podocyte permeability. For comparison, stimulated cells treated with L-NIL had significantly reduced LPS-induced permeability. * $p < 0.05$ compared to no treatment. ** $p < 0.05$ compared to LPS ($n = 5$). (B) Lucigenin luminescence ($n = 4$) and (C) supernatant NO₂ + NO₃ were measured ($n = 3$). Both NOS and NOX inhibitors reduced lucigenin luminescence (B), whereas only L-NIL reduced NO production (C). * $p < 0.05$ compared to no treatment. ** $p < 0.05$ compared to LPS.

Fig. 8.

Nitric oxide synthase decoupling does not affect LPS-induced podocyte permeability. Cells were stimulated with LPS (100 ng/ml), in the presence of BH₄ (100 μ M) for (A) 4 and (B, C) 18 h. Albumin permeability was measured using the transwell assay ($n = 4$) (A), superoxide was measured using the lucigenin assay ($n = 4$) (B), and nitric oxide was measured using a nitric oxide analyzer ($n = 3$) (C). Stimulated cells treated with BH₄ had no change in LPS-induced permeability (A). Treatment with BH₄ significantly reduced LPS-induced superoxide production (B). BH₄ had no significant effect on LPS-induced nitric oxide production (C). * $p < 0.05$ compared to no treatment. ** $p < 0.05$ compared to LPS treatment.

Fig. 9. ONOO⁻ is not the dominant reactive species associated with the leaky phenotype. (A) Unstimulated podocytes were treated with an NO donor (DETA NONOate, DETA), a ONOO⁻ donor (SIN-1), or a superoxide donor (X/XO). Cell lysates were analyzed for 3-nitrotyrosine and reported as arbitrary units (**p* < 0.05 vs SIN-1 treatment). (B) Cells were treated with LPS as for Fig. 7, with the addition of a ONOO⁻ donor (SIN-1), a ONOO⁻ scavenger (melatonin), and an NOS coupling agent (sepiapterin) (**p* < 0.05 vs no treatment). (C) Cells were again treated with LPS and analyzed for protein barrier function as for Fig. 7 with the addition of the same agents (**p* < 0.05 vs no treatment; ***p* < 0.05 vs LPS treatment).

Fig. 10.

Endogenous and exogenous nitric oxide act in a HIF-1 and CDC42/Rac1-dependent fashion to induce podocyte permeability. Cells were either (A) treated with DETA (100 μ M) or (B) stimulated with LPS (100 ng/ml) in the presence of CDC42/Rac1 inhibitor (ML141 10 μ M), HIF inhibitor (chetomin 0.15 μ M), and iNOS inhibitor (GW100 50 μ M) for 4 h. In (A) CDC42/Rac1 inhibitor and HIF inhibitor significantly reduced NO-mediated increased permeability in podocytes. iNOS inhibitor (GW100) had no significant effect. In (B) the CDC42/Rac1 inhibitor, HIF inhibitor, and iNOS inhibitor significantly reduced LPS-mediated increased permeability in podocytes. (A) * p < 0.05 compared to no treatment (n = 5). (B) * p < 0.05 compared to no treatment (n = 4).