

Nitric oxide synthase isoforms play distinct roles during acute peritonitis

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Abstract

Background. Acute peritonitis is the most frequent complication of peritoneal dialysis (PD). Increased nitric oxide (NO) release by NO synthase (NOS) isoforms has been implicated in acute peritonitis, but the role played by the NOS isoforms expressed in the peritoneum is unknown.

Methods. We investigated the structural and functional consequences of acute peritonitis induced by LPS in wild-type (WT) mice versus knockout mice (KO) for the endothelial NOS (eNOS), the inducible NOS (iNOS) or the neuronal NOS (nNOS).

Results. The level of NO metabolites (NO_x) in the dialysate was maximal 18 h after LPS injection. LPS induced a significant increase in the transport of small solutes and decreased ultrafiltration in WT mice. These changes, which occurred without vascular proliferation, were paralleled by the upregulation of nNOS and eNOS, and the induction of iNOS. The transport modifications induced by LPS were significantly reversed in eNOS KO mice, but not modified in mice lacking iNOS or nNOS. In contrast, the increase of dialysate NO_x was abolished in iNOS KO mice and significantly reduced in eNOS KO mice, but left unchanged in mice lacking nNOS. Mice lacking iNOS also showed more severe inflammatory changes, and a trend towards increased mortality following LPS.

Conclusion. These data demonstrate specific roles for NOS isoforms in the peritoneal membrane and suggest that selective eNOS inhibition may improve peritoneal transport during acute peritonitis.

Keywords: acute peritonitis; knockout mice; LPS; NO synthases; peritoneal dialysis

Introduction

Nitric oxide (NO) plays an essential role in a variety of processes including regulation of blood flow and vascular permeability, mucosal defence, leukocyte recruitment,

immune regulation and neurotransmission. Three distinct NO synthase (NOS) isoforms are involved in the synthesis of NO from L-arginine. The neuronal (nNOS, NOS1) and endothelial (eNOS, NOS3) NOS are constitutive, calcium-dependent isoforms. nNOS, which was first located in the central and peripheral nervous system, is also detected in non-neuronal cells, including myocytes, epithelial cells, mast cells and neutrophils [1–4]. eNOS, first and foremost identified in endothelial cells, is also expressed in epithelial cells, smooth muscle cells and T lymphocytes [5–7]. The calcium-independent, inducible isoform (iNOS, NOS2) is expressed in many cell types, including macrophages and endothelial cells, and is induced by inflammatory cytokines and bacterial metabolites such as lipopolysaccharide (LPS) [8].

Acute peritonitis remains the most frequent complication of peritoneal dialysis (PD) [9]. Early studies by Krediet and colleagues have shown increased NO levels in the dialysate during acute peritonitis [10,11], which prompted us to investigate the molecular basis of the production of NO in the peritoneum. We first demonstrated specific NOS activity in the peritoneal membrane, due to the expression of the three NOS isoforms [12,13]. Under baseline conditions, eNOS is the predominant NOS isoform in the peritoneum, reflecting the endothelial component of the membrane. During acute peritonitis, there is a selective upregulation of both endothelial and inducible NOS isoforms, in parallel with structural modifications that include increased endothelial area and mononuclear cell infiltrate [10,12].

The role played by NO during peritonitis has been investigated in rat and mouse models using a 6-day model of catheter-induced peritonitis [14,15]. In this model, acute peritonitis was reflected by functional modifications, including increased transport for small solutes, faster glucose reabsorption and loss of ultrafiltration (UF), which were attributed to increased effective peritoneal surface area (EPSA) due to capillary recruitment and/or vascular proliferation [10,12]. Pharmacological studies with the non-specific NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) [14] as well as studies using eNOS

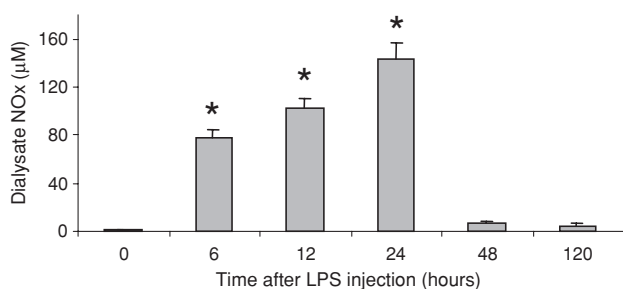


Fig. 1. Time course of dialysate NOx levels in LPS-treated mice. The total dialysate NOx (nitrate + nitrite) levels were measured using the Griess reaction. There were at least four mice at each time point. $P < 0.001$ (ANOVA); * $P < 0.05$ T_x versus T_0 .

knockout (KO) mice [15] demonstrated that the eNOS-derived NO was instrumental in the structural (vascular proliferation) and functional (hyperpermeability) modifications induced by acute peritonitis. However, these studies did not address the potential role(s) of other NOS isoforms in the peritoneal membrane and could not discriminate between the structural and functional effects in the early phase of acute peritonitis—when vascular proliferation has not yet occurred. Recent studies, based on the effect of L-NAME in a LPS-induced peritonitis mouse model [16], suggested that NO could play a role in the modifications of transport parameters in the early phase of peritonitis, in the absence of vascular proliferation. However, the latter investigation, performed in wild-type mice, could not address the contributions of the three different NOS isoforms in transport and structural changes in the peritoneum.

In the present study, we have used transgenic mouse models to provide direct evidence for a specific role of each NOS isoform in the functional changes and release of NO metabolites associated with acute peritonitis induced by LPS.

Material and methods

Animals, experimental groups and acute peritonitis model

Experiments were performed on adult male C57 BL/6J mice (Iffa Credo, Brussels, Belgium) and eNOS, iNOS and nNOS mice (all on the C57BL/6J background). Mice lacking eNOS (eNOS KO) and their wild-type littermates (eNOS WT) were generated as described previously [17] and obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The iNOS WT and KO mice [18] were obtained from P. Brouckaert (Ghent University, Ghent, Belgium). The nNOS KO and WT mice [19] were obtained from B. Casadei (University of Oxford, Oxford, UK). All animals had access to appropriate standard diet and tap water *ad libitum*. The experiments were approved by the local Ethics Committee and conducted in accord with the NIH Guide for the Care and Use of Laboratory Animals.

The first series of experiments were performed on C57 BL/6J mice, including the LPS time-course study ($n = 24$ mice) and the investigation of transport parameters and structural changes at baseline and following LPS treatment ($n = 14$ pairs of mice). In a second round of experiments, at least six pairs of eNOS mice, iNOS mice and nNOS mice were investigated during LPS-induced acute peritonitis, in comparison with WT mice under control conditions. Acute peritonitis was generated by i.p. injection of LPS (10 mg/kg, Escherichia coli serotype O111: B4, Sigma). Eighteen hours after LPS injection, all mice underwent a peritoneal equilibration test (PET) as described below.

Table 1. Clinical and biological parameters in the NOS isoform mice

NOS isoform	<i>N</i>	Body weight (g)	Haematocrit (%)	Dialysate WBC ($\times 10^4$ /mL)	MAP (mmHg)
eNOS					
WT	5	27 \pm 1	47 \pm 1	68 \pm 3	82 \pm 3
WT-LPS	5	22 \pm 0.5	47 \pm 1	138 \pm 22 ^a	78 \pm 5
KO-LPS	5	21 \pm 0.5	49 \pm 1	119 \pm 10 ^a	87 \pm 2
nNOS					
WT	5	29 \pm 0.5	46 \pm 1	68 \pm 7	82 \pm 3
WT-LPS	5	30 \pm 1	53 \pm 2	158 \pm 9 ^b	73 \pm 2
KO-LPS	4	28 \pm 0.5	46 \pm 2	219 \pm 48 ^b	75 \pm 4
iNOS					
WT	5	27 \pm 1	48 \pm 4	51 \pm 5	81 \pm 2
WT-LPS	4	28 \pm 0.5	51 \pm 2	155 \pm 11 ^c	74 \pm 2
KO-LPS	5	26 \pm 1	54 \pm 1	285 \pm 46 ^{c,d}	77 \pm 3

WBC, white blood cells; MAP, mean arterial blood pressure; WT, wild-type mice; WT-LPS, LPS-induced peritonitis in wild-type mice; KO-LPS, LPS-induced peritonitis in knockout mice.

The parameters in peritonitis groups were recorded at 18 h after LPS injection.

^a $P < 0.05$ versus eNOS WT mice; ^b $P < 0.05$ versus nNOS WT mice; ^c $P < 0.05$ versus iNOS WT mice; ^d $P < 0.05$ versus iNOS WT-LPS mice.

Peritoneal transport studies, tissue sampling

A peritoneal equilibration test (PET) was used to investigate peritoneal transport parameters in mice as previously described [20]. Briefly, after anaesthesia with ketamine and xylazine, mice were placed on a thermopad at 37°C and catheterized for blood pressure monitoring (right common carotid artery) and saline infusion (right jugular vein, 0.9% NaCl, 0.3 mL/h). After 30 min, a silicon catheter (Venflon 22 GA, Baxter) was inserted into the peritoneal cavity and 2.0 mL of 7% dialysate (Dianeal; Baxter) was instilled [20]. Blood and dialysate samples (50–100 µL) were taken from carotid artery and the PD catheter at time 0 and at 30 min, 60 min, and 120 min of dwell time. Haematocrit was measured before PD exchange. At the end of the dwell, the dialysate was recovered from the peritoneal cavity and net UF was calculated [15,20]. Dialysate white blood cells (WBCs) were counted using a haemocytometer (Marienfeld, Lauda, Germany) and the differential count (macrophages, neutrophils and lymphocytes) was established after Wright-Giemsa staining (Sigma) following the manufacturer's instruction. The mice were exsanguinated and urea, glucose, sodium and total protein were assayed using Kodak Ektachem DT60 II and DTE II analysers (Eastman Kodak Company, Rochester, NY, USA). Peritoneum samples were carefully dissected and processed for fixation and protein extraction as described previously [12–15]. Samples from the visceral and parietal peritoneum were fixed for 3 h at 4°C in 4% paraformaldehyde in a 0.1 M phosphate buffer, rinsed and embedded in paraffin. Samples from the visceral peritoneum were dissected, snap-frozen in liquid nitrogen and stored at -80°C .

Antibodies

The NOS isoforms were detected with well-characterized monoclonal antibodies against eNOS, nNOS and iNOS (Transduction laboratories, Lexington, KY) [13,15]. Other antibodies included a goat antibody against CD31 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a monoclonal antibody against β -actin (Sigma, St. Louis, MO, USA).

Immunoblotting analyses

SDS-PAGE and immunoblotting were performed as described [12,13,15]. Protein extracts from the visceral peritoneum were separated on 7.5% acrylamide slabs and transferred to nitrocellulose. Efficiency of transfer to nitrocellulose was tested by Ponceau red (Sigma) staining and immunoreactivity for β -actin. The membranes were blocked for 30 min, incubated overnight with the primary antibody at 4°C, washed, incubated for 1 h with peroxidase-labelled secondary antibodies (Dako, Glostrup, Denmark) and visualized with enhanced chemiluminescence (Amersham, Little Chalfont, UK). The immunoblots were performed at least in duplicate, and specificity of the signal was determined by comparison with positive controls. Densitometry analysis was performed with a StudioStar

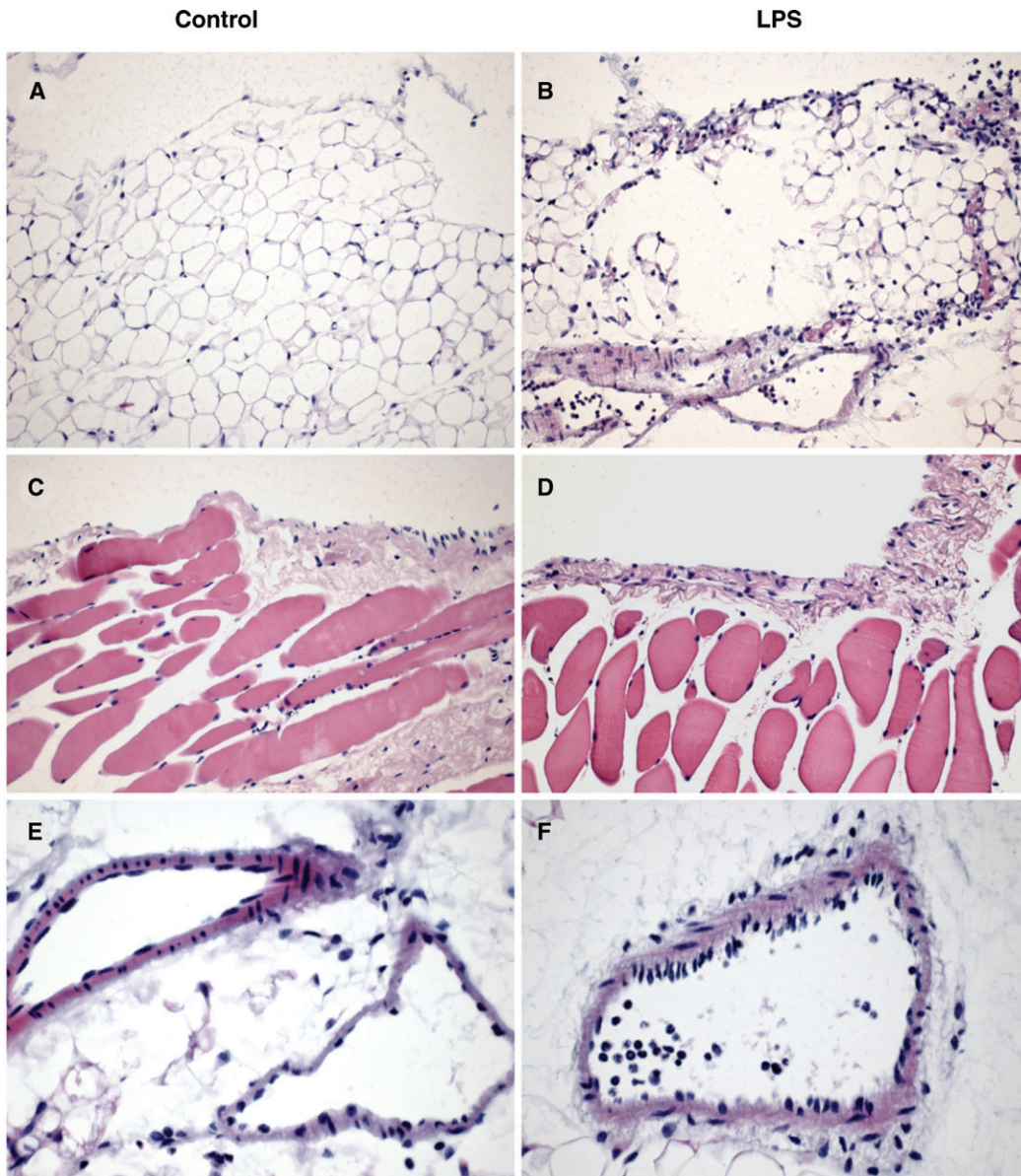


Fig. 2. Effects of LPS-induced peritonitis on the peritoneum: morphology. Haemalun–eosine staining in the visceral (A, B, E and F) and parietal (C and D) peritoneum of control (A, C and E) or LPS-treated mice (B, D and F). LPS-induced peritonitis is reflected by an inflammatory infiltrate in the visceral (B) and parietal (D) peritoneum. Marginating and perivascular leukocytes are clearly identified in LPS-treated mice (F).

Scanner (Agfa-Gevaert, Mortsels, Belgium) using the NIH-Image V1-57 software.

Tissue staining and immunohistochemistry

Haematoxylin and eosin (HE) staining and immunostaining were performed as described previously [12,14,15]. After blocking in 0.3% H_2O_2 and incubation with 10% normal serum, sections were incubated for 45 min each with the primary antibody, biotinylated IgG and avidin–biotin peroxidase (Vector Laboratories, Burlingame, CA, USA). The MOM immunodetection kit (Vector) was used for monoclonal antibodies. Immunolabelling was visualized using aminoethylcarbazole (Vector). Sections were viewed under a Leica DMR coupled with a Leica DC 300 camera (Leica, Heerbrugg, Switzerland). The specificity of immunolabelling was confirmed by incubation without primary antibody and with nonimmune IgG (Dako). Microvascular density was assessed using CD31 immunostaining, as previously reported in detail [21]. Sections were digitalized to measure the density of vascular structures (in N vessels/ mm^2) and the relative

endothelial area (i.e. the ratio between the cumulative endothelial area and the peritoneum area) as described [15,22]. All slides were coded and analysed by the same operator. The analysis included a mean of 10 fields per sample and four mice in each group. The intensity of the inflammatory reaction in the visceral peritoneum in four pairs of randomly selected iNOS mice was graded as follows: 0, absence of rolling; 1, rolling; 2, mild infiltrate (perivascular and/or interstitial); 3, severe infiltrate (perivascular and/or interstitial).

Determination of nitrite/nitrate (NOx)

The production of nitrite and nitrate in the dialysate was measured using a colorimetric assay based on the Griess reaction [15], following the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA).

Data analysis

Data are presented as mean \pm SEM. Statistical analyses were performed using ANOVA and the appropriate parametric (Bonferroni) or

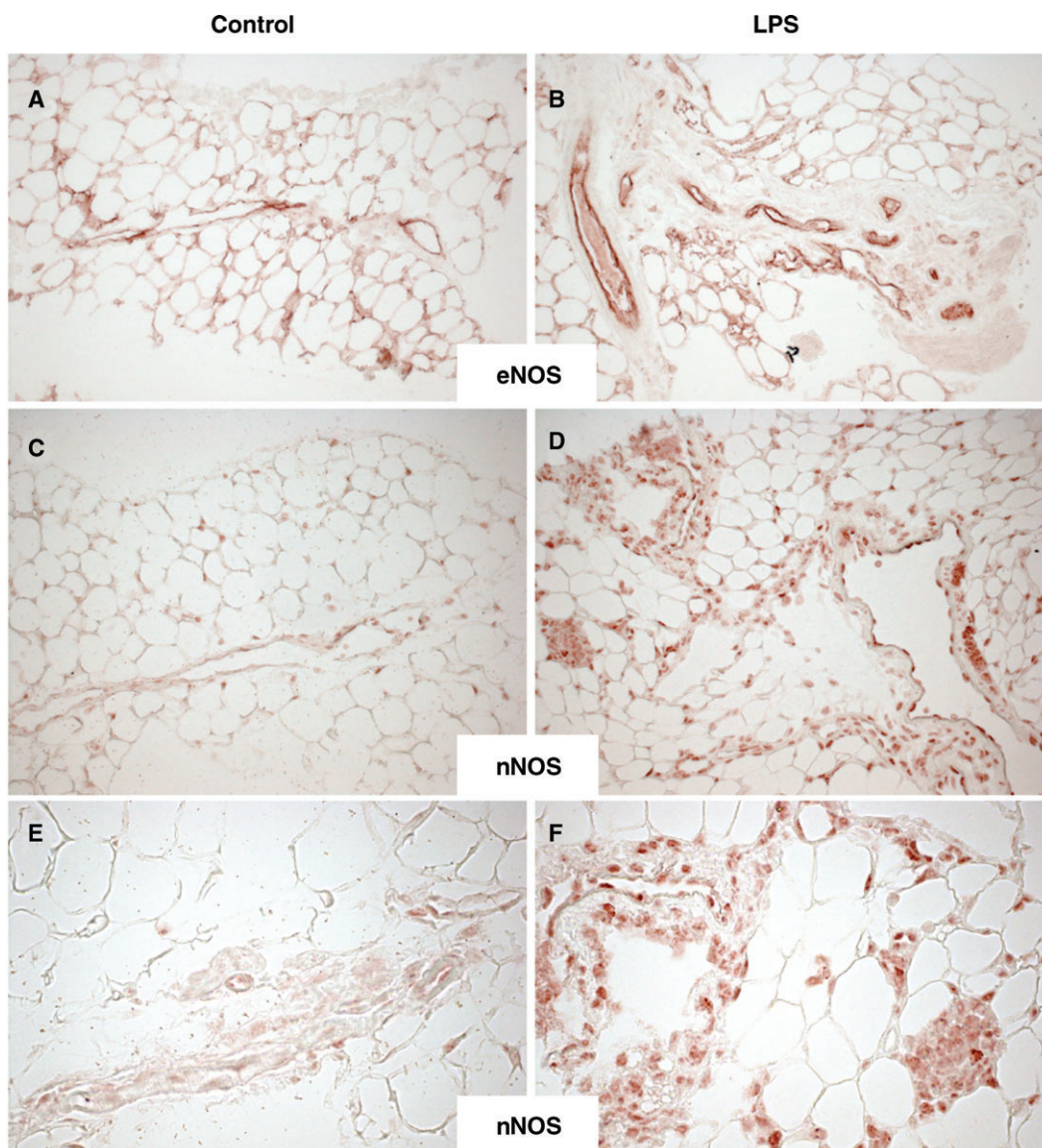


Fig. 3. LPS induction of eNOS and nNOS in the peritoneum: immunostaining. Immunostaining for eNOS (A and B) and nNOS (C–F) in the visceral peritoneum of control (A, C and E) and LPS-treated mice (B, D and F). Immunoreactivity for eNOS is located in the endothelium lining peritoneal blood vessels in control mice (A). The staining intensity of blood vessels stained for eNOS is markedly increased in LPS-treated mice with peritonitis (B). Immunoreactivity for nNOS is located in the scattered nuclei of visceral peritoneum in control mice (C) and is over-expressed in endothelial cells, nerve sections and perivascular inflammatory cells (D and F) in LPS-induced peritonitis.

non-parametric (Mann–Whitney) test for multiple comparisons (Graph-Pad Software, San Diego, CA, USA).

Results

Effect of LPS on C57 BL/6J mice

Effect of LPS on NO_x level in the dialysate: time-course analysis. Once released in biological fluids, NO is oxidized to the inactive and stable metabolites NO₂/NO₃ (NO_x), which can be quantified as a surrogate marker of NO production [23]. Treatment with LPS induced a significant increase in the NO_x level in dialysate (Figure 1). The induction was time dependent, reaching a maximum between 12

and 24 h, with a sharp decrease thereafter. Sequential analysis of the differential WBC count confirmed that this time course corresponded to a majority of macrophages ($55 \pm 2\%$ at 12 h and $50 \pm 3\%$ at 18 h, $N = 4$ mice for each time-point) in the dialysate, before the characteristic neutrophil invasion was observed after 24 h (data not shown). Accordingly, most analyses were performed 18 h after LPS treatment.

Morphological characteristics of LPS-induced peritonitis. Morphological examination of the visceral and parietal peritoneum (HE staining) showed that, in comparison with control mice, LPS-induced acute peritonitis was reflected by a discrete infiltrate of inflammatory cells in both the

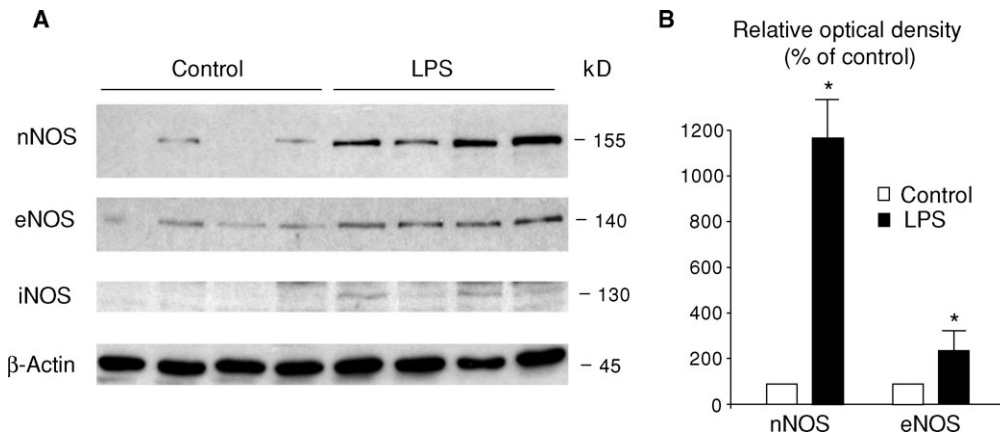


Fig. 4. LPS induction of NOS isoforms: immunoblotting. Representative immunoblots for nNOS, eNOS and iNOS in peritoneum extracts prepared from control mice and LPS-treated mice (20 μ g protein/lane). The blots were stripped and reprobed for β -actin for loading accuracy. A consistent upregulation of nNOS (155 kD) and eNOS (140 kD) is detected in LPS-treated mice. The signal for iNOS (130 kD, lower band of the blot) is not detected in control samples but appears in LPS-treated mice. Densitometry analysis confirms the major upregulation of nNOS (relative optical density: $1170 \pm 200\%$, $*P = 0.005$) and eNOS (relative optical density: $247 \pm 110\%$, $*P = 0.01$) following LPS.

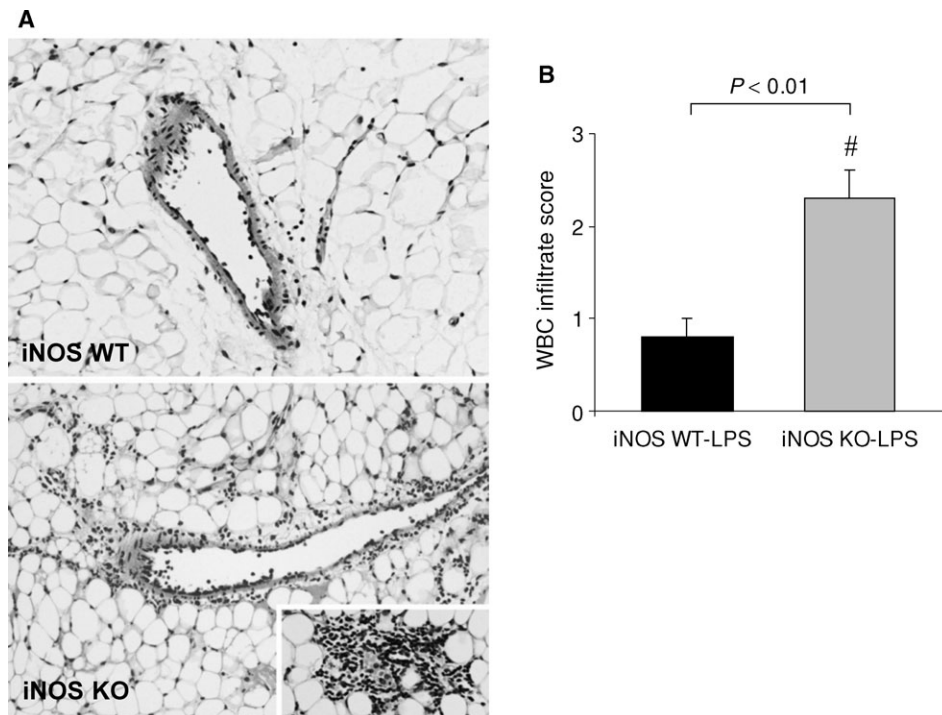


Fig. 5. Peritoneal infiltrate in iNOS mice. Haemalun–eosine staining in the visceral peritoneum (A) of LPS-treated iNOS WT mice (upper panel) and iNOS KO mice (lower panel). LPS-induced peritonitis is reflected by a discrete inflammatory infiltrate, which is markedly more severe in iNOS KO mice. The infiltrate score (B) is significantly increased in LPS-treated iNOS KO mice ($P < 0.01$ versus iNOS WT mice, $n = 6$).

visceral (Figure 2A,B) and parietal peritoneum (Figure 2C, D). The leukocytes infiltrate started at the early stage of inflammation. Six hours after LPS injection, the majority of inflammatory cells were polymorphonuclear leukocytes, mostly located in the capillary lumen. At 18 h, marginating leukocytes were observed in the capillaries (Figure 2E,F). There was no apparent difference in the nature of the leukocyte infiltrate between the three KO strains. After 48 h, the proportion of polymorphonuclear leukocytes decreased in parallel with a progressive increase in mononuclear leuko-

cytes (macrophages and lymphocytes). At 120 h after LPS injection, there were clusters of mononuclear leukocytes (data not shown).

Effect of LPS-induced peritonitis on NOS expression. Immunostaining (Figure 3) showed that LPS-induced acute peritonitis was associated with the increased eNOS signal in the endothelium lining peritoneal blood vessels (Figure 3A,B) and increased immunoreactivity for nNOS in endothelial and perivascular inflammatory cells including

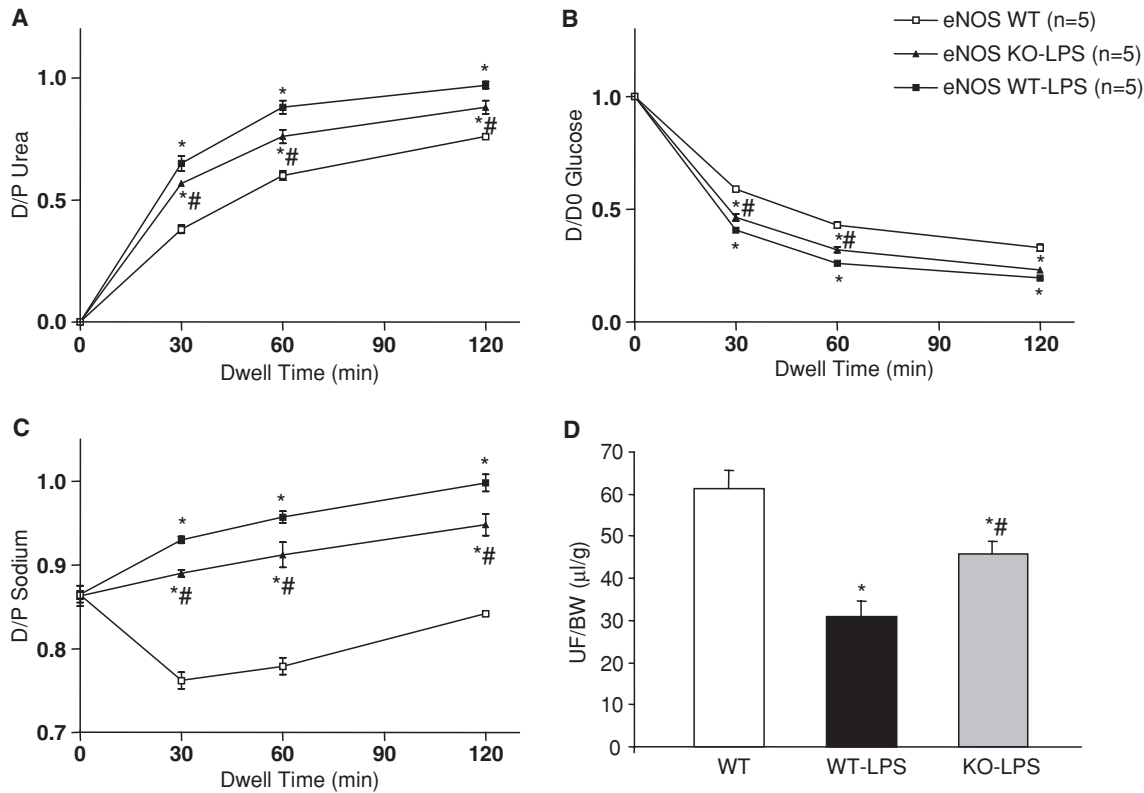


Fig. 6. PD transport parameters: effect of LPS and eNOS deletion. The dialysate-to-plasma (D/P) ratio of urea (panel A) and sodium (panel C), the progressive removal of glucose from the dialysate (D/D0 glucose; panel B) and net ultrafiltration (UF normalized to BW, panel D) were determined in control mice (WT, open squares), eNOS WT mice treated with LPS (WT-LPS, black squares) and eNOS KO mice with LPS (KO-LPS, black triangles) during a 2-h exchange. LPS treatment induces a major increase in the transport of urea and glucose, with a loss of sodium sieving and a loss of ultrafiltration. All these modifications are significantly attenuated in eNOS KO mice. **P* < 0.05 versus WT mice; #*P* < 0.05 versus eNOS WT-LPS mice.

Table 2. Water and solute transport across the peritoneum in NOS isoform mice

NOS isoform	N	Net UF/ BW (µL/g)	Na ⁺ sieving (%)	D ₃₀ /D ₀ glucose	D ₁₂₀ /D ₀ glucose
eNOS					
WT	5	61 ± 4	12 ± 1	0.59 ± 0.01	0.33 ± 0.01
WT-LPS	5	31 ± 4*	-7 ± 1*	0.41 ± 0.01*	0.19 ± 0.01*
KO-LPS	5	46 ± 3*#	-2 ± 1*#	0.46 ± 0.02*#	0.23 ± 0.01*#
nNOS					
WT	5	59 ± 4	12 ± 1	0.60 ± 0.01	0.32 ± 0.01
WT-LPS	5	24 ± 1*	-4 ± 1*	0.47 ± 0.03*	0.20 ± 0.01*
KO-LPS	4	20 ± 4*	-5 ± 2*	0.45 ± 0.02*	0.17 ± 0.01*
iNOS					
WT	5	54 ± 2	12 ± 1	0.59 ± 0.01	0.32 ± 0.01
WT-LPS	4	29 ± 1*	-4 ± 1*	0.48 ± 0.02*	0.21 ± 0.01*
KO-LPS	5	27 ± 4*	-4 ± 2*	0.49 ± 0.02*	0.21 ± 0.01*

BW, body weight; UF, ultrafiltration; D₁₂₀, dialysate glucose concentration at 120 min; D₃₀, dialysate glucose concentration at 30 min; D₀, dialysate glucose concentration before the dwell.

**P* < 0.05 versus WT mice; #*P* < 0.05 versus WT-LPS mice.

macrophages, and nerve sections (Figure 3C–F). The LPS treatment was also reflected by the induction of iNOS immunoreactivity in rare, infiltrating macrophages (data not shown). Morphometry analysis on CD31-stained sections confirmed that both the vascular density (in vessels/mm²: 58 ± 10 in control mice versus 85 ± 16 in LPS-treated mice, *P* = 0.22) and cumulative endothelial area (1.1 ± 0.4% in

control mice versus 1.6 ± 0.3% in LPS-treated mice, *P* = 0.33) were not significantly modified 18 h after LPS treatment. Immunoblotting studies (Figure 4) demonstrated that LPS-induced peritonitis was associated with a major up-regulation of nNOS (155 kD) and eNOS (140 kD), and the induction of iNOS (130 kD) in the peritoneum (Figure 4A). Densitometry analysis confirmed that LPS treatment

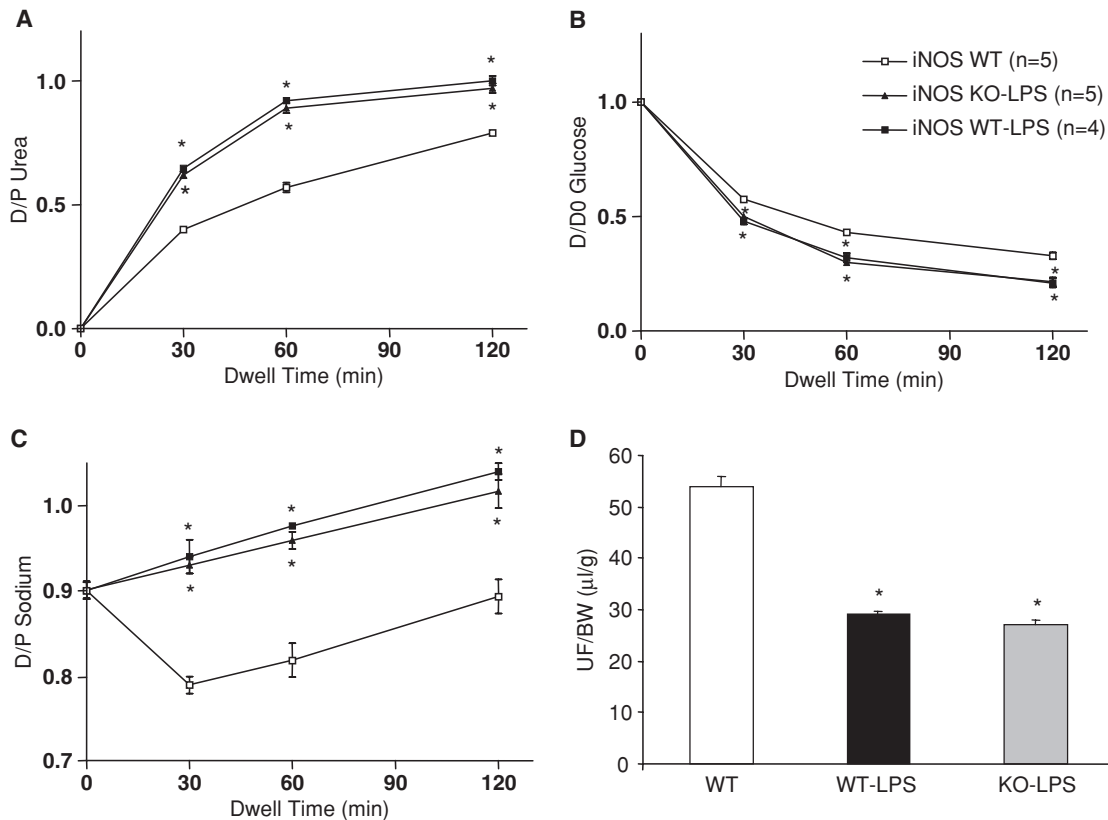


Fig. 7. PD transport parameters: effect of LPS and iNOS deletion. The dialysate-to-plasma (D/P) ratio of urea (A) and sodium (C), the progressive removal of glucose from the dialysate (D/D0 glucose, B) and net ultrafiltration (UF/BW, D) were determined in control mice (WT, open squares), iNOS WT mice treated with LPS (WT-LPS, black squares) and iNOS KO mice treated with LPS (KO-LPS, black triangles) during a 2-h exchange. Acute peritonitis induces a major increase in the urea and glucose transport, with a loss of sodium sieving and a loss of ultrafiltration. These changes are not modified in iNOS KO mice. * $P < 0.05$ versus WT mice.

induced an 11-fold increase in nNOS expression (relative optical density: $1170 \pm 200\%$, $P = 0.005$), and 2-fold increase in eNOS expression (relative optical density: $247 \pm 110\%$, $P = 0.01$) in the peritoneum (Figure 4B).

Effect of LPS on mice lacking specific NOS isoforms

Clinical and biological parameters. All mice used in the study were similar in terms of body weight and haematocrit (Table 1). Mice lacking eNOS, which have a higher mean arterial blood pressure (MAP) at baseline [15,17], showed a trend for higher MAP during acute peritonitis (Table 1). In comparison with control mice, mice with LPS-induced peritonitis were characterized by cloudy dialysates and significantly increased WBC counts. Among the LPS-treated mice, mice lacking iNOS showed significantly higher WBC counts in the dialysate versus their wild-type littermates (Table 1). Further examination of the visceral peritoneum (Figure 5) revealed that the extent of inflammation induced by LPS was significantly higher in iNOS KO versus WT mice (inflammation score: 2.3 ± 0.3 versus 0.8 ± 0.2 , $N = 5$, $P < 0.01$). These modifications were not observed in the eNOS and nNOS mice. The iNOS KO mice were also characterized by a higher mortality rate following LPS injection (mortality rate: 1/6 in iNOS WT versus 4/10 in iNOS KO mice).

Transport parameters in mice: effects of specific NOS deletion. The effects of LPS-induced peritonitis on transport parameters were investigated in NOS KO mice, using a 2-h PD exchange (Figures 6–8, Table 2). This protocol induced a progressive increase in the dialysate-to-plasma ratio for urea (panel A), a progressive reabsorption of glucose from the dialysate (panel B), a fall in the dialysate-to-plasma ratio of sodium during the first 30 min of the dwell (sodium sieving; panel C) and a net UF (panel D) as previously reported [20]. Acute peritonitis induced by LPS caused a major increase in the small solute transport (urea and glucose), with a loss of sodium sieving and a loss of UF in WT-LPS mice. All these modifications were significantly attenuated in eNOS KO mice, with a partial recovery of sodium sieving and UF, and a reduced transport for small solutes (Figure 6, Table 2). In strong contrast, the LPS-induced transport modifications were not significantly modified in mice lacking iNOS (Figure 7) or nNOS (Figure 8) (see also Table 2).

Effects of specific NOS deletion on dialysate NO_x levels. Treatment with LPS induced a 15- to 30-fold increase in the dialysate NO_x levels in all types of WT mice (Figure 9). The LPS-induced increase in NO_x was significantly attenuated in eNOS KO mice (average: -50%) and almost abolished (average: -97%) in iNOS KO mice. These strong effects

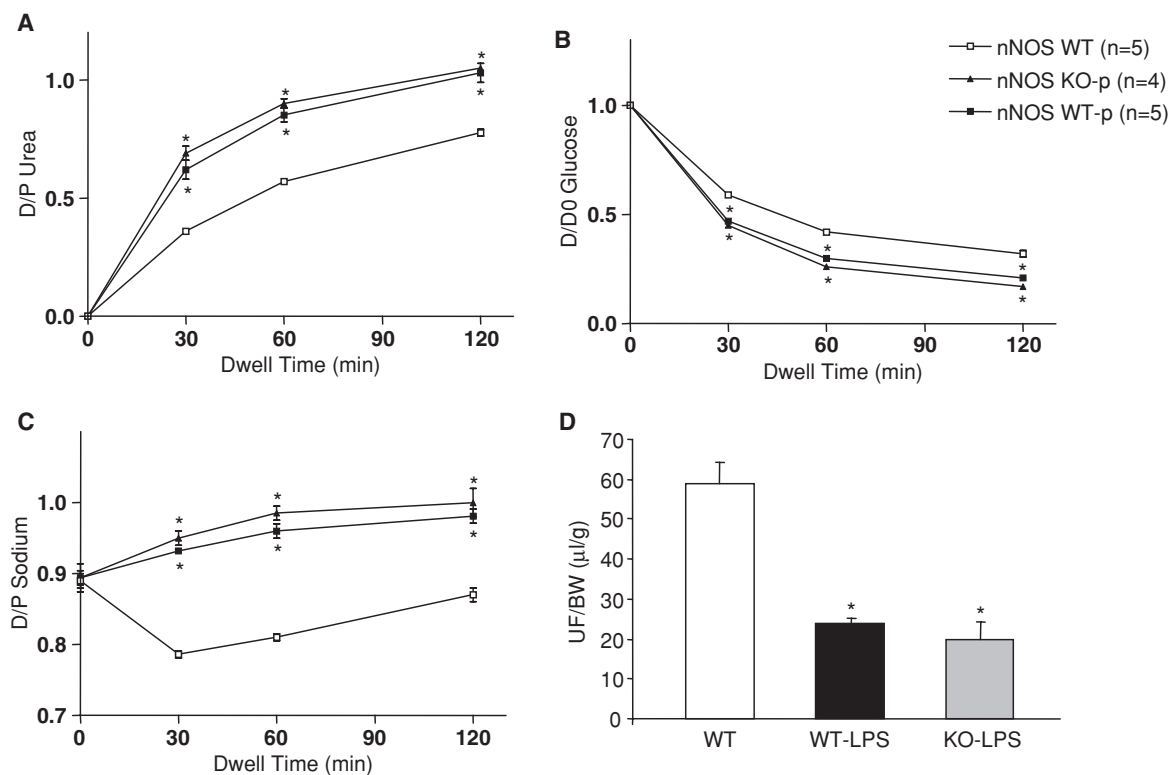


Fig. 8. PD transport parameters: effect of LPS and nNOS deletion. The dialysate-to-plasma (D/P) ratio of urea (A) and sodium (C), the progressive removal of glucose from the dialysate (D/D0 glucose, B) and net ultrafiltration (UF/BW, D) were determined in control mice (WT, open squares), nNOS WT mice treated with LPS (WT-LPS, black squares) and nNOS KO mice with LPS (KO-LPS, black triangles) during a 2-h exchange. Acute peritonitis induces a major increase in the urea and glucose transport, with a loss of sodium sieving and a loss of ultrafiltration. These changes are not modified in nNOS KO mice. * $P < 0.05$ versus WT mice.

contrasted with a mild, non-significant decrease (average: -14%) in nNOS KO mice.

Discussion

In the present study, we provide direct evidence suggesting that each NOS isoform plays a distinct role in the peritoneal membrane during acute peritonitis. These data are based on the differential expression of the NOS isoforms at baseline and during LPS-induced peritonitis, and on the characterization of clinical, biochemical, structural and transport parameters in wild-type mice versus mice lacking eNOS, iNOS and nNOS.

Several lines of evidence, obtained in rodent models and PD patients, show that the three NOS isoforms are differentially regulated according to the underlying pathogenic process [24]. A significant upregulation of eNOS alone has been documented in the peritoneal membrane exposed to long-term PD [25,26]. In contrast, catheter-induced peritonitis is reflected by a major increase in both eNOS and iNOS [12–15], whereas chronic uraemia induces changes in the expression of eNOS and nNOS [27]. The tightly regulated pattern of expression of NOS isoforms and the pleiotropic effects of NO raised the question of the role played by this complex system in the peritoneal membrane [28]. Using mouse models, we previously demonstrated the crucial role of eNOS in the structural modifications (infil-

trate, vascular proliferation) and transport changes in acute peritonitis [15,20]. However, these data were obtained using a 6-day model of catheter-induced bacterial peritonitis in the single eNOS KO mouse. In contrast, the present study was designed to decipher the specific roles of the three NOS isoforms during the early phase of LPS-induced acute peritonitis.

Intraperitoneal administration of LPS, an integral component of the outer membrane of Gram-negative bacteria, initiates a rapid, coordinated recruitment and activation of leukocytes, followed by overproduction of proinflammatory mediators and NO [29]. Mice injected with LPS showed cloudy dialysates with increased WBC counts and NO_x levels, inflammatory cell infiltrates in the peritoneum, a major upregulation of NOS and increased transport of small solutes with ensuing UF failure. These changes, which are similar to those documented in other models [12–16], are characteristic of acute peritonitis in PD patients [10,11]. The time of investigation, 18 h after LPS injection, corresponds to the maximal release of NO. Addition of the NOS inhibitor L-NAME at that time point significantly reversed transport modifications and NO_x release in this model [16]. Taken together, these results suggested that the excessive release of NO may be involved in the early changes during peritonitis. However, because NOS inhibitors lack specificity, it has not been possible to characterize the role of each NOS isoform in this acute phase of inflammation/infection. Thus, transgenic mice lacking

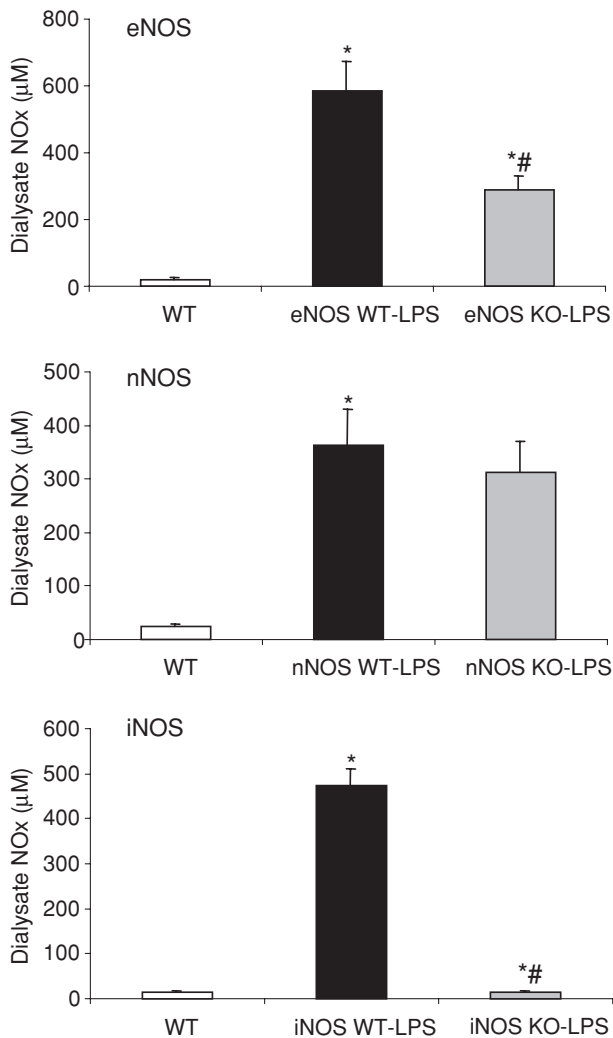


Fig. 9. Dialysate NOx levels: effect of eNOS, nNOS and iNOS deletion. Treatment with LPS in WT mice induced a significant increase in NO metabolites (NOx) in the dialysate. This effect was significantly reversed in eNOS KO mice (top panel) and almost abolished in iNOS KO mice (bottom panel), whereas it was unchanged in nNOS KO mice (middle panel). * $P < 0.05$ versus WT mice; # $P < 0.05$ versus WT-LPS mice. $N = 4$ in each group.

NOS isoforms provide an excellent tool to answer that question, using established procedures for investigation of the peritoneal membrane [20].

Endothelium-derived NO is a potent vasodilator that participates in the regulation of blood pressure, vascular permeability, angiogenesis and leukocyte adhesion [30–32]. We have shown previously that mice lacking eNOS are protected against functional and structural modifications in a 6-day model of peritonitis [15]. The present data confirm and extend these findings, by showing that the lack of eNOS significantly attenuates the acute increase in the small solute transport induced by LPS, resulting in a significant improvement in UF. Of note, the deletion of eNOS does not totally reverse transport changes, as it does not abolish the burst of NOx release in the dialysate. This may be related to the uneven effect of LPS on the specific NOS regulation: the 2.5-fold increase in eNOS is below the

induction of iNOS (normally absent from the peritoneum) and the >10-fold increase in nNOS. The lack of total reversibility could also be due to pro-inflammatory cytokines such as IL-6 liberated during inflammation [33] or, alternatively, to post-translational modifications of target proteins. At variance with the 6-day catheter peritonitis model, the upregulation of eNOS 18 h after LPS does not reflect a significant vascular proliferation in the peritoneum. This important difference illustrates the fact that eNOS, initially described as a constitutive isoform, can actually be induced by a variety of stimuli including growth factors, cytokines and bacterial LPS [2,34]. At any rate, the upregulation of eNOS is critical to regulate transport parameters in the early phase of peritonitis, which emphasizes the therapeutic potential of selective inhibitors targeting this isoform.

The induction of iNOS by cytokines and proinflammatory agents is known to increase NO production during many inflammatory responses, including peritonitis [12]. In particular, LPS bind to their CD14 receptor on the surface of macrophages, resulting in the transcriptional induction of iNOS with subsequent release of NO [35]. In turn, the generation of large quantities of NO by activated macrophages may contribute to circulatory failure and organ injury. However, enhanced NO release may also exert beneficial effects, including a bactericidal effect and augmentation of host defence [36,37]. Although it has been reported that iNOS-deficient mice exhibit enhanced leukocyte–endothelium interactions in endotoxaemia [38] and that iNOS activity plays a protective role in response to inflammation [39], the role of iNOS during acute peritonitis has not been addressed. The present study indicates that induction of iNOS in the peritoneal membrane is a major mechanism for the increased release of NO metabolites during the early phase of LPS peritonitis. Mice lacking iNOS showed a nearly complete abolition of NOx release in the dialysate despite unchanged transport parameters, which provides a strong argument for the local NO activity rather than increased transport from the circulation [11]. Of note, the abolition of NOx release may also reflect the fact that the exasperated inflammation due to the lack of iNOS may trigger the release of cytokines (including TNF α) down-regulating the expression of eNOS [40]. Furthermore, the lack of iNOS was reflected by higher WBC counts in the dialysate, more severe inflammatory changes in the tissue and a trend towards increased mortality following LPS treatment. Accordingly, it is tempting to propose that the release of NO mediated by iNOS exerts a protective role in acute peritonitis. This protective role may be related to host-defence mechanisms, independently of changes in transport parameters.

The nNOS isoform plays an important role in the generation of NO for neuronal development, maturation and neurotransmission [23,41]. nNOS is also expressed in extraneuronal cells, including neutrophils [42], endothelial cells [43] and vascular smooth muscle cells [44]. In the peritoneum, nNOS is expressed in nerves and peritoneal arteries, where it could play a role in uraemic neuropathy and vasculopathy [27]. We demonstrate a 10-fold increase in nNOS expression in the peritoneum following LPS. Like eNOS, the expression of the ‘constitutive’ nNOS can be regulated in response of neuronal cells to stress [2]. The strong induction observed here appears to be related either

to the time course or the nature of the stimulus. Indeed, the 6-day model of catheter-induced peritonitis does not induce nNOS expression [12] whereas LPS treatment induces nNOS expression in muscles [45]. The role of nNOS in the peritoneum is distinct from that of eNOS and iNOS, with a minimal effect on NO_x release, and no reversibility on transport parameters. These differences may reflect expression in distinct cellular compartments. Alternatively, one could hypothesize that the deletion of nNOS may induce compensatory upregulation of other NOS isoforms in the peritoneal membrane [46]. A trend for increased dialysate WBC counts was observed in nNOS KO mice, supporting the hypothesis that nNOS deficiency may promote leukocyte rolling and adhesion [47]. However, at variance with iNOS, this was not reflected by changes in the severity of inflammatory reaction to LPS. By analogy with other models, one could hypothesize that nNOS may regulate inflammatory pain [48] during acute peritonitis.

In summary, we have shown that the LPS-induced acute peritonitis in the mouse is reflected by the upregulation of the three NOS isoforms. The transport alterations induced by peritonitis were reversed in eNOS KO mice, whereas the inflammatory changes were more severe in iNOS KO mice. These data give new insights in the differential roles of NOS isoforms in the peritoneal membrane and suggest that selective eNOS inhibition may improve peritoneal transport during acute peritonitis, whereas non-specific NOS inhibitors may impair host-defence mechanisms [49,50].

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