Nephrol Dial Transplant (2008) 23: 3485–3493
doi: 10.1093/ndt/gfn266
Advance Access publication 14 May 2008

Original Article

NDT
Nephrology Dialysis Transplantation

The uraemic toxin phenylacetic acid impairs macrophage function

Sven Schmidt[1], Timm H. Westhoff[1], Philipp Krauser[1], Ralf Ignatius[2], Joachim Jankowski[1], Vera Jankowski[1], Walter Zidek[1] and Markus van der Giet[1]

[1]Charité Centrum 10-Nephrology and [2]Institut für Mikrobiologie und Hygiene, Charité-Campus Benjamin Franklin, Berlin, Germany

Abstract

Background. Nitric oxide (NO) is known to be an important mediator of macrophage cytotoxicity. NO in macrophages is generated via the inducible nitric oxide synthases (iNOS). Macrophage dysfunction is an important contributory factor for the increased incidence of infections in uraemia. Recently, we identified phenylacetic acid (PAA) as a novel uraemic toxin in patients on regular haemodialysis. PAA inhibits iNOS expression. In the present study, we investigated the impact of PAA on macrophage function.

Methods. RAW 264.7 cells were stimulated by LPS/IFN-γ in the absence and presence of PAA. iNOS mRNA was determined by real-time PCR, iNOS protein was examined by western blotting and the NO degradation product, nitrite, by Griess assay. Macrophage phagocytosis was assessed by FACS and fluorescence microscopy. Further we quantified the cytotoxicity against intracellular bacteria (*Salmonella typhimurium)* by a macrophage-killing assay. ELISA and Bioplex protein array system was used for the investigation of iNOS second messenger pathways (NF-κB, ERK1/2, JNK and p38MAPK). iNOS mRNA half-lifetime in the presence or absence of PAA was determined by real-time PCR.

Results. PAA significantly inhibits iNOS mRNA induction in RAW 264.7 cells by LPS/IFN-γ [6 h: LPS/IFN-γ-stimulation: 100%; LPS/IFN-γ-stimulation/PAA (1 mM): 68 ± 7%] at concentrations comparable to those of patients on chronic haemodialysis. iNOS protein expression and nitrite formation in RAW 264.7 cells were significantly inhibited by PAA. iNOS mRNA half-lifetime was not affected by PAA. The phagocytic activity of RAW 264.7 was not significantly affected by PAA, whereas the cytotoxicity against intracellular bacteria was significantly reduced. Analysis of the iNOS signal transduction pathways provided evidence that activation of the mitogen-activated kinases ERK1/2 and JNK is significantly blocked by PAA, whereas activation of p38MAPK is unaffected. The NF-κB pathway was not affected by PAA.

Conclusions. The present findings show that the uraemic toxin PAA has inhibitory effects on macrophage-killing function, which are mediated by inhibitory effects on transcriptional iNOS regulation. iNOS inhibition by PAA might affect immunoregulatory processes and could play a role in aggravation of immunodeficiency of patients with end-stage renal disease.

Keywords: iNOS; macrophage function; phenylacetic acid; uraemic toxin

Introduction

Infections are one of the major causes of disease, hospitalization and death in patients with end-stage renal disease (ESRD) [1]. The infection-related hospitalization rate is known to be 235/1000 patient-months in adult dialysis patients with an in-hospital lethality of 20–30% [2]. The increased incidence of infectiological events in ESRD patients results from a host of factors, including cannulation of the arteriovenous fistula [3], bacterial affinity to a foreign material such as PTFE grafts and catheters [4], the dialyzers' impact on the complement system [5], deficiency and resistance to vitamin D as an immunomodulatory agent [6], malnutrition [7] and the assumed immunomodulatory impact of a variety of uraemic toxins [1]. The mechanisms underlying the immunomodulatory effects of uraemic toxins remain largely elusive.

Macrophage function is a crucial prerequisite for both specific and unspecific immune response. Macrophages are supposed to be dysfunctional in uraemia but the underlying mechanisms remain to be determined. One essential event in the bactericidal function of macrophages is the generation of reactive nitrogen intermediates (RNI) produced by the inducible nitric oxide synthases (iNOS). RNI include nitric oxide and its physiological metabolites. RNI are potent antimicrobial agents in murine and human macrophages [8]. Numerous studies correlated the levels of RNI in macrophages with their antibacterial action to mycobacteria [9].

Recently we identified phenylacetic acid (PAA) as a novel uraemic toxin in humans with ESRD [10]. We showed that this substance is a potent inhibitor of iNOS induction in macrophages. *In vivo* plasma concentrations of PAA have

Correspondence and offprint requests to: Sven Schmidt, Charité-Campus Benjamin Franklin, Charité Centrum 10-Nephrology, Hindenburgdamm 30, Berlin 12200, Germany. Tel: +49-30-8445-2217; Fax: +49-30-8445-4235; E-mail: s.schmidt@charite.de

been estimated previously by our group. In ESRD patients a plasma concentration of 3.49 ± 0.33 mM was measured, whereas in young, healthy patients PAA was not detectable [10]. Former research of our group showed that 69.8% of the PAA is non-protein bound. Thus, the biologically active concentration is 2.44 mM [10]. In the present work, we investigated the effect of PAA on macrophage function with special regard to the bactericidal function of macrophages. We show that PAA reduces cytotoxicity of macrophages. In addition, we investigated the underlying pathways of the PAA-induced iNOS inhibition.

Methods

Preparation and stimulation of RAW 264.7 cells

The murine macrophage cell line RAW 264.7 was obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). Cells were kept and cultured as previously described [10]. Cells were plated at a concentration of 100 000 cells/ml and used for the experiments when they reached 80% confluence. Cells were then stimulated for different periods in a humidified incubator at 37°C and 5% CO_2 with 1-μg/ml LPS (serotype 0111:B4 from *E. coli*; Sigma-Aldrich, Taufkirchen, Germany) together with 100 U/ml IFN-γ (Sigma-Aldrich, Taufkirchen, Germany). Cells were serum starved in a serum-reduced medium for 24 h before use.

Preparation of RNA and real-time PCR

The design of the primers and probes for murine iNOS/β-actin, the RNA extraction, and the quantitative real-time PCR (RT-PCR) were performed as previously described [10].

iNOS protein analysis

Preparation of proteins from RAW 264.7 cells and western blot analysis were performed as previously described [10].

Measurement of nitrite formation

Measurement of nitrite production as an assay of NO release was carried out using the Griess reaction. Aliquots of the culture medium were mixed with an equal volume of Griess reagent [1% sulphanilamide/0.1% *N*-(1-napthyl)ethylenediamine dihydrochloride in 5% phosphoric acid], the mixture was incubated at room temperature for 10 min and the absorbance at 540 nm was measured using a photometer (iEMS reader; Labsystems, Helsinki, Finland). Standard curves were constructed using known concentrations of sodium nitrite.

iNOS mRNA half-lifetime

RAW 264.7 cells were stimulated with LPS (1 μg/ml) and IFN-γ (100 U/ml) for 6 h in the presence or absence of PAA (1 mM, 5 mM). For determination of iNOS mRNA half-life, transcription was inhibited by the addition of 10 mg/ml actinomycin D (Act D). Cells were harvested for RNA isolation every 2 h for 6 h as described above. iNOS and β-actin mRNA were measured by RT-PCR. iNOS mRNA levels were normalized against β-actin to determine half-lifetime.

Fluorescence microscopy

RAW 264.7 cells were cultured on 8-well chamber slides (Nunc, Germany) in the RPMI 1640 medium (10% FCS) to subconfluency. Cells were starved for 24 h. After incubation with LPS (1 μg/ml) and IFN-γ (100 U/ml) for 6 h in the presence or absence of different concentrations of PAA (1 mM, 5 mM), the cells were treated with a 1:1000 diluted fluorescent latex particles (FLP) suspension (Fluoresbrite YG carboxylate microspheres, diameter 2 μm, Polyscience Inc., Warrington, PA, USA) at 37°C for 1 h. After phagocytosis, cells were washed three times with cold PBS to remove noningested beads. The extent of phagocytosis was monitored by using an inverted microscope (Axiovert 200M, Zeiss, Oberkochen, Germany) equipped for fluorescence microscopy (excitation 470 nm, emission 525 nm).

Quantification of phagocytosis by FACS analysis

RAW 264.7 cells were cultured in 12-well plates to 80% confluence. Quiescent cells (24 h) were incubated with LPS (1 μg/ml) and IFN-γ (100 U/ml) for 6 h in the presence or absence of various concentrations of PAA (1 mM, 5 mM). After incubation, the cells were treated with 20 μl of a mixture of 0.2 ml of FLP suspension and 1.8 ml of PBS at 37°C for 1 h. For negative control, cells were kept at 4°C for 1 h. After treatment cells were washed with ice-cold PBS. The cells were dissociated with Trypsin/EDTA at 37°C for 5 min. The cells were centrifuged at 200 *g* for 5 min, the supernatant was removed and cells were suspended in FACS buffer (PBS, containing 0.5% BSA, 2 mM EDTA). Cell suspensions were analysed by the flow cytometer BD FACScalibur (Becton Dickinson Labware, Lincoln Park, NY, USA). The RAW 264.7 cell population was gated by height and granularity by forward and side scatter, respectively. The gated cells were analysed by the fluorescence histogram. The phagocytic activity was determined as the ratio of RAW 264.7 cells that ingested at least one FLP, per 50 000 RAW 264.7 cells.

Macrophage-killing assays

100 000 RAW 264.7 cells/well were cultured in RPMI 1640 (Biochrom, Berlin, Germany) and 1% FCS without antibiotics in 96-well flat-bottom trays (Nunc, Roskilde, Denmark) at 37°C and 5% CO_2. Twenty-four hours later, PAA was added at 1 mM or 5 mM, and control cells were kept in the medium. After 12 h of incubation *Salmonella enterica* serovar Typhimurium opsonized by 10%, mouse serum was added ($3–5 \times 10^6$ bacteria/well). The plates were centrifuged for 30 min at 1800 rpm and room temperature and incubated for another 15 min at 37°C to enhance the internalization of bacteria by the cells. The wells were washed three times with a medium containing 10 μg/ml gentamycin (Biochrom, Berlin, Germany), incubated for 90 min in the presence of 50 μg/ml gentamycin, and washed another three times. PAA was added again at the same concentrations,

and the cells were incubated at 37°C and 5% CO_2. To determine the numbers of initially internalized bacteria, cells from some wells were lysed after 3 h with 0.2% Triton X-100/PBS (Sigma), while the rest of the cells were kept for 24 h at 37°C before cell lysis. Bactericidal effects by the Triton X-100 concentration used had been excluded before. We have performed a serial dilution of the cell lysates. The lysates were diluted 1:100, 1:1000 and 1:10 000, respectively. One hundred microlitres of each diluted lysate was plated on a blood agar and incubated at 37°C for 24 h. The blood agar with CFUs in the range up to 500 was counted manually, and the initial bacterial number was calculated according to the dilution factor. The results are expressed as percentages of *viable* bacteria on the basis of CFU obtained at 3 and 24 h after infection. Supernatants were collected from all wells immediately before lysis and plated on blood-agar plates to exclude considerable quantities of extracellular bacteria. Numbers of extracellular bacteria did not exceed 10–30 CFU/well at 3 h and 30–100 CFU/well at 24 h after infection, while numbers of intracellular bacteria were in a range of 1–4 $\times$ 10^4 CFU/well.

Preparation of nuclear extracts

Cells were harvested, washed in ice-cold PBS and disrupted by repeated freezing and thawing cycles. Lysis was performed by hypotonic buffer (10 mM HEPES-KOH, 10 mM KCl, 1 mM DTT, 0.1 mM EGTA, IGEPAL and a protease inhibitor cocktail). After 10 min of centrifugation (10 000 *g*), supernatant, containing cytosolic protein, was discarded, and the pellet, containing the nuclei, was resuspended in a hypertonic nucleus lysing buffer (20 mM HEPES-KOH, 400 mM NaCl, 50 mM DTT, 1 mM EGTA, 25% Glycerin, IGEPAL and a protease inhibitor cocktail). After 30 min of centrifugation (13 000 *g*), nuclear protein, present in the supernatant, was collected and quantified.

Assessment of nuclear NF-κB p65

Cells were stimulated as described above for various time points with 1 μg/ml LPS and 100 U/ml IFN-γ to determine the time response of NF-κB activation in RAW 264.7 cells. For the time of maximal stimulation, cells were stimulated in the presence of different concentrations of PAA. Nuclear protein was extracted. Nuclear NF-κB p65 was assessed using a commercially available ELISA kit (EZ-Detect Transcription Factor for NF-kB p65, Pierce) according to manufacturer's instructions. Chemiluminescence was detected using a luminometer (Mithras, Berthold, Bad Wildbad, Germany).

Preparation of phosphoprotein and Bioplex protein array system

Cells were stimulated with LPS (1 μg/ml) and IFN-γ (100 U/ml) for different time points in the presence or absence of different concentrations of PAA (1 mM, 5 mM). For negative control, untreated cells were used. Cells were washed with ice-cold PBS to stop stimulation. Cells were harvested and lysed in a phosphoprotein-lysis-buffer (BioRad, Munich, Germany) containing protease and phosphatase inhibitors. Cells were disrupted by sonication. After centrifugation (4500 *g*, 20 min, 4°C), the supernatant containing the phosphoprotein was collected.

Different phosphokinases were measured in triplicates using the Bioplex protein array system (BioRad, München, Germany), according to the manufacturers' protocol. The system is a multiplexed, particle-based, flow cytometric assay that utilizes anti-phosphokinases monoclonal antibodies linked to microspheres incorporating distinct proportions of two fluorescent dyes. Phosphorylation levels of ERK1/2, JNK and p38MAPK could be detected. For negative control, untreated cells were used.

Patients. To determine the effect of plasma of ESRD patients on iNOS mRNA expression, we collected plasma of healthy and ESRD patients.

The study has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association and has been approved by the local ethical committee, and informed consent was obtained. Ten patients with ESRD undergoing maintenance haemodialysis for 27 $\pm$ 8 months were investigated. Patients were stable and free from intercurrent illness (age: 52 $\pm$ 7 years) and in a good state of health. All patients were routinely dialyzed for 5 h three times weekly using biocompatible polyamix haemodialysis membranes (Polyflux 17L, Gambro Dialysatoren GmbH, Hechingen, Germany). Plasma from patients with end-stage chronic renal failure was obtained before and after the regular haemodialysis session. Blood was taken from the arterial site of the haemodialysis fistula and immediately centrifuged at 4000 *g* and 20°C for 5 min. Plasma from 10 healthy controls (age: 47 $\pm$ 9 years) was taken as controls. RAW 264.7 cells were cultured and stimulated with LPS (1 μg/ml) and IFN-γ (100 U/ml) in the presence of 50% serum of healthy or ESRD patients. iNOS mRNA was determined as described above.

NMR spectroscopy

To quantify PAA concentrations in the plasma of the control and ESRD patients before and after dialysis, proton-NMR (1H-NMR) measurements were carried out using a Bruker AMX 400 FT spectrometer (Bruker Analytische Messtechnik GmbH, Rheinstetten, Germany) operating at a field strength of 400 MHz as described previously by our group [10].

Statistics. Data were analysed using Prism 4.02 (GraphPad Software Inc., San Diego, CA, USA). Data are presented as mean $\pm$ SEM. Differences were analysed for significance using the non-parametric Mann–Whitney *U*-test. $P < 0.05$ was considered significant.

Results

PAA inhibits cytokine-induced iNOS expression

Data on iNOS expression and production of nitrite in RAW 264.7 stimulated with LPS have been published previously by our group [10] and were confirmed in the present

analysis. Stimulation of RAW 264.7 cells with 1 μg/ml LPS and 100 U/ml IFN-γ induced a time-dependent increase in mRNA expression, protein expression and production of nitrite. iNOS mRNA expression increased significantly after 2 h of stimulation with a maximum after 6 h of stimulation (data not shown). Protein expression of iNOS was found after 12 h and had a maximum after 24 h (data not shown). Nitrite formation in RAW 264.7 cells showed a maximum after 24 h of stimulation (data not shown). To investigate the inhibition by PAA on iNOS mRNA/protein expression and nitrite formation, PAA was applied at different concentrations before stimulation. PAA inhibited stimulated iNOS mRNA expression dose dependently in RAW 264.7 cells after 6 h (Figure 1A). iNOS protein expression in RAW 264.7 cells stimulated for 12 h with LPS/IFN-γ was also significantly reduced in the presence of PAA (1 mM and 5 mM) [Figures 1B & C; LPS/IFN-γ: 100% and LPS/IFN-γ+ PAA (1 mM): 59 ± 5%]. Lastly, there was a significantly lower nitrite production in RAW 264.7 cells after 24 h of stimulation with LPS/IFN-γ in the presence of PAA (1 mM) (Figure 1D; LPS/IFN-γ: 100% and LPS/IFN-γ+PAA (1 mM): 72 ± 8%).

Effect of PAA on iNOS mRNA degradation

We investigated the effect of PAA on mRNA degradation to evaluate potential interactions. The half-lifetime of iNOS mRNA was measured after addition of Act D by RT-PCR. The half-life of iNOS mRNA induced by LPS and IFN-γ alone (4.68 h) was not significantly ($P < 0.05$) different from the half-life of iNOS mRNA in the presence of PAA (1 mM: 4.70 h; 5 mM: 4.94 h) (Figure 2).

Effect of PAA on phagocytosis activity of RAW 264.7 cells

We tested the influence of PAA on phagocytosis of RAW 264.7 cells. To determine whether PAA inhibits phagocytosis, we measured the rate of internalization of latex beads in RAW 267.4 cells (Figure 3A). The amount of ingested latex beads per cell was measured using FACS analysis. We could demonstrate that LPS/IFN-γ stimulation for 6-h significantly increased ($P < 0.05$) phagocytosis of latex beads. The presence of PAA (1 mM or 5 mM) did not affect the LPS/IFN-γ-induced increase in phagocytosis of RAW 267.4 cells (Figure 3B).

Effect of PAA on macrophage-killing function

Next, we measured the survival of *Salmonella enterica* serovar Typhimurium in untreated RAW 267.4 macrophages or macrophages pre-treated with various concentrations of PAA for 12 h. Twenty-four hours after infection numbers of intracellular bacteria were significantly ($P < 0.05$) increased in the PAA (1 mM and 5 mM)-treated cells as compared to untreated cells (Figure 3C; control: 100.2 ± 6.3%; 1 mM PAA: 201.9 ± 30.0%; 5 mM PAA: 194.9 ± 17.4%) ($n = 6$ experiments).

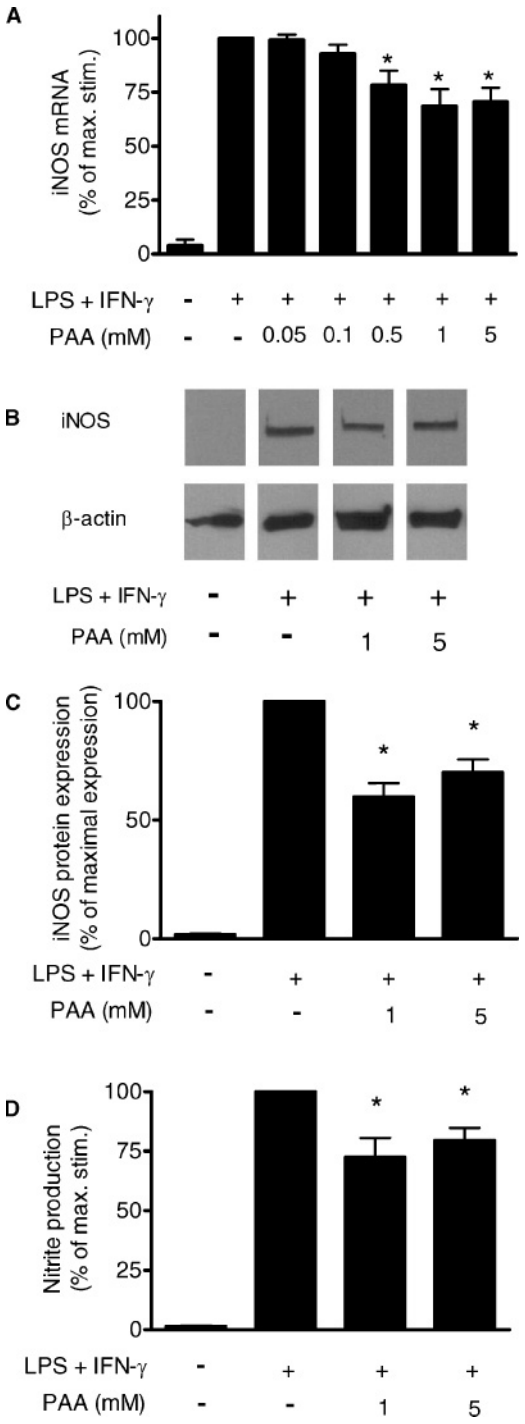

Fig. 1. Expression of iNOS measured by real-time PCR, protein blotting of iNOS or nitrite formation in RAW 264.7 cells. (**A**) The iNOS expression was measured by real-time PCR after 6 h of stimulation by LPS (1 μg/ml) and IFN-γ (100 U/ml) and in the presence of various PAA concentrations. Stimulated iNOS expression was set to 100% ($n = 6$ individual experiments). $^*P < 0.05$ compared to stimulation. (**B**) Representative protein blotting of iNOS and β-actin after 12 h of stimulation of RAW 264.7 cells with LPS (1 μg/ml) and IFN-γ (100 U/ml) and in the presence of PAA (1 mM, 5 mM). (**C**) Signals of iNOS were quantified and normalized to those of β-actin using a bioimaging analyzer. Data represent means of triplicate determinations from each of four protein preparations. $^*P < 0.05$ compared with stimulation ($n = 6$ individual experiments). (**D**) The effect of PAA (1 mM, 5 mM) on LPS/IFN-γ-induced (24 h) nitrite production in RAW 264.7 cells. Data are means ± SEM ($n = 6$ individual experiments). $P < 0.05$ compared with LPS/IFN-γ stimulation.

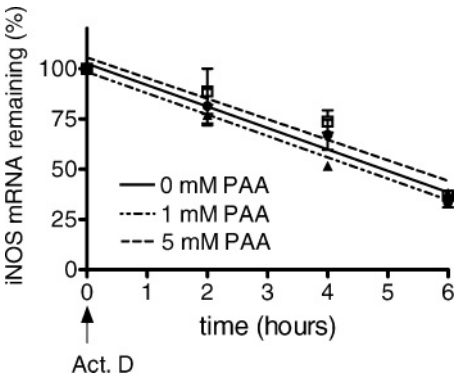

Fig. 2. mRNA iNOS half-lifetime. RAW 264.7 cells were stimulated with LPS (1 μg/ml) and IFN-γ (100 U/ml) for 6 h in the presence or absence of PAA (1 mM, 5 mM). Transcription was inhibited by addition of 10 mg/ml actinomycin D (Act D). iNOS and β-actin mRNA were measured by RT-PCR every hour. iNOS expression after 6 h was set as 100%. iNOS mRNA levels were normalized against β-actin. Shown is the decrease in iNOS mRNA every hour ($n = 6$ experiments).

Effect of PAA on phosphorylation of MAP kinases

To determine the effect of PAA on the phosphorylation of MAP kinases (MAPKs), we used a bead-based phosphorylation assay of the three major MAPKs: JNK, ERK1/2 and p38MAPK. The kinases play an essential role in the induction of iNOS by LPS and IFN-γ stimulation. First we stimulated RAW 264.7 cells with LPS (1 μg/ml) and IFN-γ (100 U/ml) for various time points. Total protein was extracted and the phosphorylation of different MAPKs was evaluated. Figure 4A shows a time-dependent phosphorylation of ERK1/2 with a maximum at 30 min. JNK showed maximal phosphorylation at 30 min after stimulation (Figure 4B). The time-dependent phosphorylation of p38MAPK has also a maximum of stimulation at 30 min (Figure 4C). Total ERK1/2, JNK and p38MAPK were not influenced by LPS and IFN-γ stimulation (data not shown). Next, RAW 264.7 cells were preincubated with different concentrations of PAA (1 mM, 5 mM). After 30 min of stimulation with LPS and IFN-γ, total MAPK and phosphorylated MAPK were determined. Preincubation with 5 mM PAA significantly reduces the phosphorylation of ERK1/2 [LPS/IFN-γ (control): 8.2 ± 1.0-fold; LPS/IFN-γ+PAA (5 mM): 4.4 ± 1.0-fold; $P < 0.05$ versus control, $n = 9$] (Figure 4D). As shown in Figure 4E, PAA significantly reduced cytokine-induced phosphorylation of JNK [LPS/IFN-γ (control): 23.5 ± 1.3-fold; LPS/IFN-γ+PAA (5 mM): 9.2 ± 1.0-fold; $P < 0.05$ versus control, $n = 9$] (Figure 4E). Total JNK and ERK1/2 were not influenced by PAA (data not shown). The total amount and phosphorylation status of p38MAPK were not significantly influenced by PAA (Figure 4F).

Influence of PAA on NF-κB activation

To verify the effect of PAA on NF-κB activation, NF-κB p65 was measured by ELISA in nuclear extracts. Nuclear NF-κB p65 was stimulated by LPS (1 μg/ml) and IFN-γ (100 U/ml) in a time-dependent manner (Figure 5A). The findings are in accordance with former results of Kim *et al*.

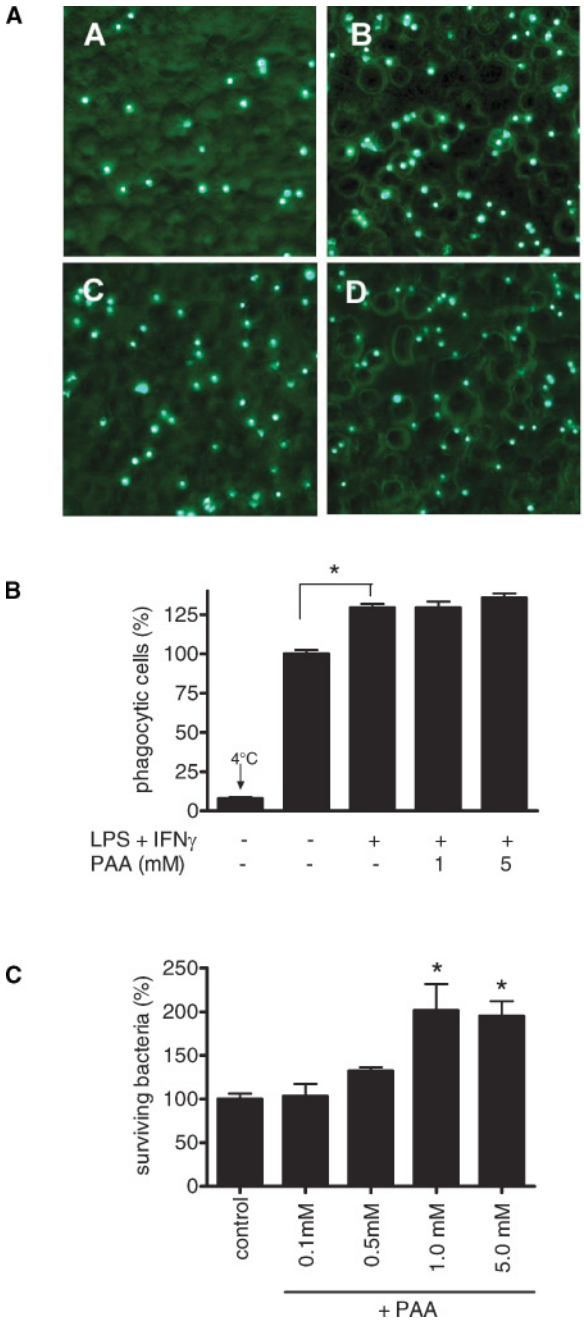

Fig. 3. PAA does not inhibit phagocytosis of macrophages but increases surviving of intracellular bacteria. (**A**) RAW 264.7 cells were stimulated with LPS (1 μg/ml) and IFN-γ (100 U/ml) for 6 h in the presence or absence of PAA (1 mM, 5 mM). Cells were then treated with fluorescent latex particles (FLP). Ingestion of FLP was monitored by using an inverted microscope (Axiovert 200M, Zeiss, exc: 470 nm; em: 525nm). A = unstimulated RAW cells, B = stimulated RAW cells, C and D = stimulated RAW cells + PAA (C = 1 mM and D = 5 mM). (**B**) RAW 264.7 cells were stimulated with LPS (1 μg/ml) and IFN-γ (100 U/ml) for 6 h in the presence or absence of PAA (1 mM, 5 mM). Cells were then treated with FLP and ingestion of particles was measured by a flow cytometer (BD FACScalbiur). Phagocytic cell activity of each sample is shown by percentage compared with control cells (unstimulated). $^*P < 0.05$ compared to unstimulated cells ($n = 6$ individual experiments). (**C**) RAW 264.7 cells were cultured for 6 h in the presence or absence of PAA. *S. typhimurium* was opsonized and allowed to internalize for 30 min. After 24 h, survival of bacteria was measured. Surviving of bacteria in the absence of PAA (control) was set to 100%. $^*P < 0.05$ compared to control ($n = 6$ individual experiments).

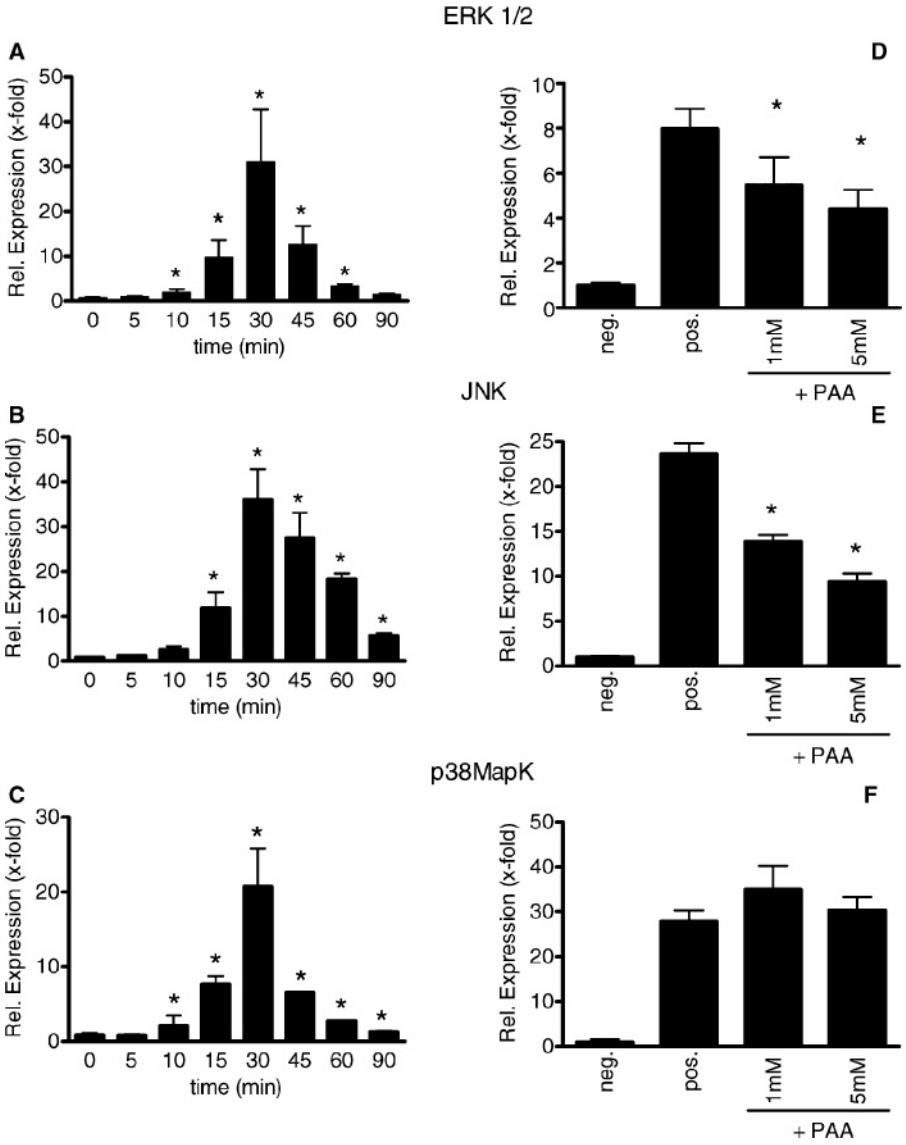

Fig. 4. PAA inhibits ERK1/2 and JNK activation. (**A**–**C**) RAW 264.7 cells were stimulated with LPS (1 μg/ml) and IFN-γ (100 U/ml) and time-dependent activation of ERK1/2 (**A**), JNK (**B**) and p38MAPK (**C**) was measured using the Bioplex protein array system. $^*P < 0.05$ compared to unstimulated cells (0 min). $n = 4$ experiments each. (**D**–**F**) RAW 264.7 cells were stimulated with LPS (1 μg/ml) and IFN-γ (100 U/ml) for 30 min in the presence or absence (pos.) of PAA (1 mM, 5 mM) to measure phosphorylation of ERK-1/2 (**D**), JNK (**E**) and p38MAPK (**F**) was measured using the Bioplex protein array system. $^*P < 0.05$ compared to positive control. $n = 9$ experiments each.

We found a time-dependent stimulation of nuclear NF-κB p65, stimulated by LPS (1 μg/ml) and IFN-γ (100 U/ml), during the first hour of stimulation (Figure 5A), in accordance with former results by Kim *et al*. [11]. Cotreatment with different concentrations of PAA did not significantly influence cytokine-induced NF-κB p65 content in the nucleus after 1 h (Figure 5B). Thus, PAA has no influence on NF-κB activation in RAW 264.7 cells.

Effect of plasma from haemodialysis patients on iNOS expression in RAW 264.7 cells

Plasma from healthy subjects did not affect iNOS expression in RAW 264.7 cells stimulated with LPS and IFN-γ (6 h). In the presence of plasma (50%) from patients with ESRD on regular haemodialysis before haemodialysis session, iNOS mRNA expression was significantly reduced ($P < 0.05$, $n = 10$ patients) (Figure 6). Post-dialysis serum inhibited iNOS mRNA expression to a significantly lower extent compared to pre-dialysis serum ($P < 0.05$, $n = 10$ patients) (Figure 6). The concentration of PAA in the plasma of the ESRD patients could be determined as 4.6 ± 0.7 mM pre-dialysis and 2.0 ± 1.6 mM post-dialysis, and the concentration of the healthy controls was 0.15 ± 0.25 mM.

Discussion

Recently it was shown that the uraemic toxin PAA is involved in the regulation of iNOS in RAW 264.7 cells by

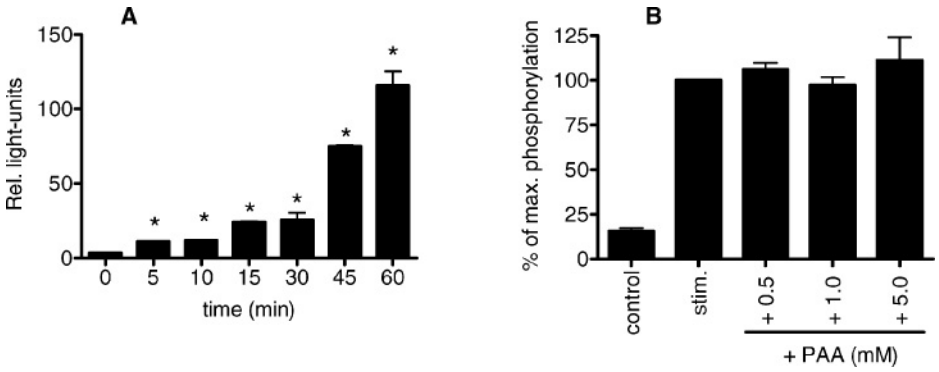

Fig. 5. PAA does not inhibit NF-κB p65 translocation. RAW 264.7 cells were stimulated for various time points with 1 μg/ml LPS and 100 U/ml IFN-γ to determine NF-κB activation by measuring nuclear NF-κB p65 using a commercially available NF-κB p65 ELISA kit. $^*P < 0.05$ compared to control (0 min). $n = 5$ experiments. (**B**) RAW 265.7 cells were stimulated for 60 min with 1 μg/ml LPS and 100 U/ml IFN-γ to determine NF-κB activation by measuring nuclear NF-κB p65 in the absence or presence of PAA. $^*P < 0.05$ significant change versus stimulation without PAA. $n = 8$ experiments.

iNOS mRNA inhibition. The influence on antimicrobial properties of macrophages and the mechanism of iNOS inhibition by PAA remains elusive. The aim of the present study was the investigation of the immunomodulatory properties of PAA and the identification of the underlying mechanism in CRF patients.

PAA causes an impaired macrophage function. The phagocytosis capacity of RAW 264.7 was not reduced by PAA, as shown in our phagocytosis experiments. The macrophage-killing assay reveals a reduced capacity for killing of ingested bacteria. The iNOS activation is reduced in a dose-dependent manner as shown in quantitative RT-PCR, western blotting and nitric measurements. Plasma from ESRD patients with an active PAA concentration of 2.3 mM (50% serum with a total serum PAA concentration of 4.6 mM) also inhibited iNOS mRNA expression, stimulated by LPS and IFN-γ. With regard to the present findings, it appears possible that PAA is responsible for this effect, although a contribution of other uraemic toxins cannot be excluded.

The activation of iNOS is based on two major cascades, the activation of NF-κB by phosphorylation and degradation of I-κB, followed by nuclear translocation of p65/p50 and phosphorylation of the MAPKs ERK1/2 and JNK, respectively. p38MAPK has iNOS inhibitory properties. Our ELISA and protein array system experiments demonstrate that the nuclear translocation of p65 is not influenced by PAA. Thus NF-κB activation is not the target of iNOS inhibition by PAA. The protein array system experiments of MAPKs phosphorylation show that JNK and ERK activation by phosphorylation is inhibited by PAA in a dose-dependent manner. Furthermore, these experiments reveal that the phosphorylation of the third major MAPK p38MAPK was not influenced by PAA. A decreased JNK and ERK phosphorylation leads to a reduced formation of AP-1 and a reduced iNOS transcription due to lacking AP-1 binding site activation.

A toxic effect of PAA resulting in a reduced cell viability was considered and ruled out to be a potential explanation for the inhibition of iNOS in former works [10]. Considering mRNA degradation as a possible mechanism of iNOS inhibition, our results demonstrate that, crucially, the half-lifetime of iNOS mRNA was not influenced by PAA. Thus, the observed iNOS inhibition cannot be explained by an increased degradation of iNOS mRNA in the presence of PAA.

The RAW cell line is derived from the monocyte system. Monocytes are phagocytic cells that produce and release ROS and RNS in response to phagocytosis or stimulation with various endogeneous and exogeneous agents. NADPH oxidase is responsible for the initial ROS production, or so-called respiratory burst. It yields to a rapid processing of internalized bacteria, protozoen, helminths, viruses and tumour cells in the phagosomes [8]. RNS production is generated by iNOS. It is capable of inducing a sustained pacteriostatic phase [12]. A combined effect of NADPH oxidase and iNOS is the formation of $ONOO^-$ by $NO^\cdot$ and O_2^- [8].

In the case of iNOS inhibition by PAA, one main mechanism of processing ingested bacteria is decreased. RNS production as well as $ONOO^-$ formation is decreased by lacking iNOS activation. This might play a role in the defence and containment of bacterial infection, especially by facultative intracellular bacteria. It is well known that CRF patients have increased mortality by infections. Haemodialysis patients have an up to 16 times increased incidence of tuberculosis (TB) [13]. At least part of this increased risk of TB is caused by an increase in the likelihood of progression from silent form to active form, because of the impaired cell-mediated immunity in CRF patients [14]. The reasons for this are mostly unknown. Expression of iNOS by macrophages is associated with the TH1-dependent killing of intracellular microbes (e.g. cryptococci, Toxoplasma, mycobacteria and Leishmania) [15]. In the murine model of TB, as a classical example of TH1-mediated immune reaction, NO plays an essential role in the killing of *mycobacterium tuberculosis* by mononuclear phagocytes [9,16,17]. In a mouse strain with genetically disrupted iNOS (iNOS−/−), infection with *M. tuberculosis* was associated with a significant higher risk of dissemination and mortality compared with the wild type C57BL/6 mice [16,18]. Similar results exist for infection with *Leishmania major* and *Klebsiella pneumoniae* [15,19].

Can the results obtained in murine macrophages be transferred to human conditions? We have previously

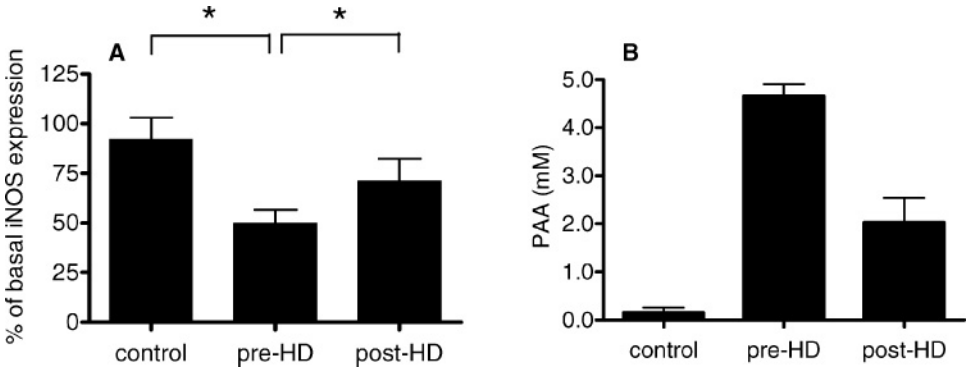

Fig. 6. Plasma from patients with end-stage renal failure inhibits iNOS mRNA expression measured by real-time PCR. (**A**) The iNOS expression was measured by real-time PCR after stimulation of RAW 264.7 cells by LPS (1 μg/ml) and IFN-γ (100 U/ml) and in the presence of plasma from healthy patients (control, $n = 10$) and plasma from patients with end-stage renal disease on chronic haemodialysis before and after haemodialysis session (HD, $n = 10$). Stimulated iNOS expression was set to 100% ($n = 10$). $^*P < 0.05$ significant change compared to stimulation. All experiments were performed as triplicates. (**B**) The concentration of PAA in plasma of healthy and ESRD patients before and after haemodialysis was measured by proton-NMR measurements ($^*P < 0.05$, $n = 10$).

demonstrated that PAA and uraemic plasma can inhibit iNOS expression in human monocytes [10]. The investigation of NO and iNOS protein in human macrophages *ex vivo* was not feasible. Whereas macrophages that are obtained from patients with inflammatory or infectious disease invariably express iNOS, peripheral blood-monocyte-derived macrophages that are obtained from normal donors and stimulated *in vitro* generally do not [20]. Furthermore, *in vitro* cultures of human macrophages lack tetrahydrobiopterin (BH_4), an essential cofactor for iNOS catalytic activity that is not constitutively expressed in human macrophages [21]. *In vivo* human macrophages may obtain BH_4 from other neighbouring cells capable of synthesizing it, such as activated lymphocytes and endothelial cells [21], but *in vitro* this is not possible. In function of the limitations of *in vitro* systems for human-macrophage differentiation and immunologic activation, the murine cell line RAW 264.7 is frequently used to elucidate iNOS regulation [22,23].

CRF patients show a decreased phagocytic capacity of neutrophils and monocytes [25]. Several uraemic toxins have been isolated and characterized, being involved in polymorphonuclear leukocytes' (PMNLs) dysfunction in uraemic patients, like granulocyte inhibiting protein I (GIPI), granulocyte inhibiting protein II (GIPII), degranulation inhibiting protein I (DIPI), degranulation inhibiting protein II (DIPII), immunuglobulin light chain and chemotaxis inhibiting protein [26].

In addition to the actions of iNOS-derived RNS and $ONOO^-$ in phagocytosis, iNOS acts as a mediator in several T-cell immunoregulatory pathways. iNOS-derived NO is required for the functional maturation of NK cells *in vivo*. NK-cell cytotoxic activity in iNOS−/− mice is almost unmeasurable in mixed lymph note cells, whereas phenotypic development of NK cells is not affected [27]. iNOS inhibition by PAA might affect immunoregulatory processes and could play a role in aggravation of immunodeficiency of CVD patients.

With regards to former investigations on iNOS regulation in the central nervous system, crucial differences of iNOS regulation RAW 264.7 cells become evident [28]. Pahan showed in rat primary astrocytes that PAA inhibits iNOS expression via reduced NF-κB activation [28]. Potential effects of PAA on MAPKs were not investigated. Conclusively, the second messenger cascade of iNOS regulation varies essentially in different cell types. Differential regulatory properties in different tissues enlarge this tissue-specific second messenger pattern. A tissue-specific functionality has been shown in NADPH oxidase as well. NADPH oxidase activation is based on a membrane assembly of several subunits, including gp91phox, p22phox, p47phox, p67phox, rac and mox [29]. The tissue specific properties of the enzyme in endothelial cells, vascular smooth muscle cells and granulocytes are a consequence of a cell-specific combination of subunits [29]. Thus, one enzyme can meet the metabolic requirements of different cell types. With regards to our results, it may be speculated that iNOS activation is modulated in diverse cell types by cell-specific activation of different second messenger pathways. Further investigations will have to be done to investigate second messenger pathways in varies cell types.

Conclusion

The present study reveals that the uraemic toxin PAA has an inhibitory effect on macrophage-killing function. The effect is mediated via inhibitory effects on transcriptional iNOS regulation by a reduced phosphorylation of ERK1/2 and JNK. iNOS inhibition by PAA might influence immunoregulatory processes and could play a role in aggravation of immunodeficiency of patients with ESRD.

Acknowledgements. We are grateful to Ursula Rüschendorf for expert technical assistance. This study was supported by a grant of the Fresenius Stiftung, Germany (MvdG, JJ) and the Sonnenfeld-Stiftung, Berlin, Germany.

Conflict of interest statement. None declared.

References

1. Vanholder R, Van Biesen W. Incidence of infectious morbidity and mortality in dialysis patients. *Blood Purif* 2002; 20: 477–480
2. Chavers BM, Solid CA, Gilbertson DT *et al*. Infection-related hospitalization rates in pediatric versus adult patients with end-stage renal disease in the United States. *J Am Soc Nephrol* 2007; 18: 952–959
3. Allon M, Depner TA, Radeva M *et al*. Impact of dialysis dose and membrane on infection-related hospitalization and death: results of the HEMO Study. *J Am Soc Nephrol* 2003; 14: 1863–1870
4. Vanholder V, Hoenich N, Ringoir S. Morbidity and mortality of central venous catheter hemodialysis: a review of 10 years' experience. *Nephron* 1987; 47: 274–279
5. Dhondt AW, Vanholder RC, Waterloos MA *et al*. Leukocyte CD14 and CD45 expression during hemodialysis: polysulfone versus cuprophane. *Nephron* 1996; 74: 342–348
6. Liu PT, Stenger S, Li H *et al*. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science* 2006; 311: 1770–1773
7. Churchill DN, Taylor DW, Cook RJ *et al*. Canadian hemodialysis morbidity study. *Am J Kidney Dis* 1992; 19: 214–234
8. MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. *Annu Rev Immunol* 1997; 15: 323–350
9. Chan J, Xing Y, Magliozzo RS *et al*. Killing of virulent Mycobacterium tuberculosis by reactive nitrogen intermediates produced by activated murine macrophages. *J Exp Med* 1992; 175: 1111–1122
10. Jankowski J, Van Der Giet M, Jankowski V *et al*. Increased plasma phenylacetic acid in patients with end-stage renal failure inhibits iNOS expression. *J Clin Invest* 2003; 112: 256–264
11. Kim SG, Kim HJ, Choi SH *et al*. Inhibition of lipopolysaccharide-induced I-kappaB degradation and tumor necrosis factor-alpha expression by dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylene dioxybiphenyl-2,2′-dicarboxylate (DDB): minor role in hepatic detoxifying enzyme expression. *Liver* 2000; 20: 319–329
12. Vazquez-Torres A, Jones-Carson J, Mastroeni P *et al*. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis: I. Effects on microbial killing by activated peritoneal macrophages *in vitro*. *J Exp Med* 2000; 192: 227–236
13. Garcia-Leoni ME, Martin-Scapa C, Rodeno P *et al*. High incidence of tuberculosis in renal patients. *Eur J Clin Microbiol Infect Dis* 1990; 9: 283–285
14. Touraine JL, Touraine F, Revillard JP *et al*. T-lymphocytes and serum inhibitors of cell-mediated immunity in renal insufficiency. *Nephron* 1975; 14: 195–208
15. Diefenbach A, Schindler H, Donhauser N *et al*. Type 1 interferon (IFNalpha/beta) and type 2 nitric oxide synthase regulate the innate immune response to a protozoan parasite. *Immunity* 1998; 8: 77–87
16. MacMicking JD, North RJ, LaCourse R *et al*. Identification of nitric oxide synthase as a protective locus against tuberculosis. *Proc Natl Acad Sci USA* 1997; 94: 5243–5248
17. Chan J, Tanaka K, Carroll D *et al*. Effects of nitric oxide synthase inhibitors on murine infection with Mycobacterium tuberculosis. *Infect Immun* 1995; 63: 736–740
18. Adams LB, Dinauer MC, Morgenstern DE *et al*. Comparison of the roles of reactive oxygen and nitrogen intermediates in the host response to Mycobacterium tuberculosis using transgenic mice. *Tuber Lung Dis* 1997; 78: 237–246
19. Tsai WC, Strieter RM, Zisman DA *et al*. Nitric oxide is required for effective innate immunity against Klebsiella pneumoniae. *Infect Immun* 1997; 65: 1870–1875
20. Fang FC. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat Rev Microbiol* 2004; 2: 820–832
21. Bertholet S, Tzeng E, Felley-Bosco E *et al*. Expression of the inducible NO synthase in human monocytic U937 cells allows high output nitric oxide production. *J Leukoc Biol* 1999; 65: 50–58
22. Chan ED, Riches DW. IFN-gamma+LPS induction of iNOS is modulated by ERK, JNK/SAPK, and p38(mapk) in a mouse macrophage cell line. *Am J Physiol Cell Physiol* 2001; 280: C441–C450
23. Kim YM, Lee BS, Yi KY *et al*. Upstream NF-kappaB site is required for the maximal expression of mouse inducible nitric oxide synthase gene in interferon-gamma plus lipopolysaccharide-induced RAW 264.7 macrophages. *Biochem Biophys Res Commun* 1997; 236: 655–660
24. Chan ED, Chan J, Schluger NW. What is the role of nitric oxide in murine and human host defense against tuberculosis? Current knowledge. *Am J Respir Cell Mol Biol* 2001; 25: 606–612
25. Muniz-Junqueira MI, Braga Lopes C, Magalhaes CA *et al*. Acute and chronic influence of hemodialysis according to the membrane used on phagocytic function of neutrophils and monocytes and pro-inflammatory cytokines production in chronic renal failure patients. *Life Sci* 2005; 77: 3141–3155
26. Haag-Weber M, Cohen G, Horl WH. Clinical significance of granulocyte-inhibiting proteins. *Nephrol Dial Transplant* 2000; 15(Suppl 1): 15–16
27. Bogdan C, Rollinghoff M, Diefenbach A. The role of nitric oxide in innate immunity. *Immunol Rev* 2000; 173: 17–26
28. Pahan K, Sheikh FG, Namboodiri AM *et al*. Lovastatin and phenylacetate inhibit the induction of nitric oxide synthase and cytokines in rat primary astrocytes, microglia, and macrophages. *J Clin Invest* 1997; 100: 2671–2679
29. Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* 2000; 86: 494–501

Received for publication: 5.11.07
Accepted in revised form: 17.4.08