Inhibition of nitric oxide synthase expression by PPM-18, a novel anti-inflammatory agent, *in vitro* and *in vivo*

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We studied the effect of PPM-18, a chemically synthesized naphthoquinone derivative and also an anti-inflammatory agent, on the lipopolysaccharide (LPS)-activated inducible NO synthase (iNOS) expression in rat alveolar macrophages. Pretreatment of macrophages with PPM-18 (0.1–10 μM) significantly inhibited nitrite production, iNOS protein expression and iNOS mRNA accumulation. PPM-18 did not directly affect the enzymic activities of iNOS and other constitutive NOS forms. The LPS-induced increase in nuclear transcription factor κB (NF-κB) p65 and p50 in nucleus was suppressed by PPM-18 (10 μM). Moreover electrophoretic mobility-shift assays demonstrated that PPM-18 inhibited DNA binding to NF-κB induced by LPS in whole cells but not when added in the nuclear extract, suggesting that PPM-18 did not interfere directly with the binding of NF-κB to DNA and that some events had to be processed before NF-κB could bind DNA. Examination of NF-κB showed that PPM-18 stabilized the NF-κB inhibitor, IκBα, by preventing its degradation from NF-κB. Therefore the stabilization of IκBα might have contributed to the inhibition of NF-κB activation. These results also indicate strongly that NF-κB is involved in the production of NO on stimulation by LPS. PPM-18 significantly decreased the production of tumour necrosis factor α in response to LPS. PPM-18 protects mice against LPS-induced lethal toxicity. These results also indicate that PPM-18 is a potent inhibitor of iNOS expression by blocking the binding of NF-κB to promoter and exerts a beneficial effect in the mouse model of sepsis.

INTRODUCTION

NO is an important messenger molecule involved in the regulation of many physiological processes [1]. NO is synthesized from the terminal guanidino nitrogen atoms of L-arginine by the action of a group of enzymes designated NO synthases (NOSs) [2]. NO formed by the constitutive isoform of NOS in the central and peripheral nervous system (nNOS) is a neurotransmitter [3]. NO generated by the constitutive isoform of NOS (eNOS) in the vascular endothelium is involved in the regulation of blood pressure and inhibits the adhesion of platelets to the endothelial surface [1,2]. Another isoform of NOS is an inducible form (iNOS) that is expressed in various cells, including macrophages, by a variety of agents such as lipopolysaccharide (LPS), tumour necrosis factor α (TNF-α) and interleukin-1 (IL-1) [4]. The cytotoxicity of NO from activated macrophages has a role in their antimicrobial activity [2]. However, excessive formation of NO after the induction of iNOS contributes importantly to the circulatory failure in septic shock of various aetiologies [5,6]. Septic shock is a major cause of death in patients in intensive care units and ranks 13th in the causes of death overall in the U.S.A. [7]. However, non-isoform-selective inhibition of NO formation might lead to side effects such as negation of various physiological actions of NO by inhibiting the activity of the constitutive isoforms of NOS. Thus a selective inhibitor of iNOS expression might be of great therapeutic potential.

The mechanism by which LPS activates macrophages is still poorly understood. A portion of the 5′ flanking region of the murine iNOS gene has been cloned [8–10]. The promoter of the murine iNOS gene contains a TATA box and a consensus sequence for the binding of transcription factors associated with stimuli that induce the expression of iNOS [9]. A functional analysis of the iNOS promoter revealed two important regions. These regions were responsive to LPS and contain a putative binding site for nuclear transcription factor κB (NF-κB) [10]. NF-κB participates in the regulation of multiple cellular genes involved in the inflammatory responses [11]. An inhibitor of NF-κB, pyrrolidine dithiocarbamate (PDTC) [12], has been shown to suppress the induction of iNOS expression in cytokines-stimulated macrophages [13,14]. Xie et al. [15] further proposed that NF-κB is a major transcription factor for iNOS gene expression. Griscavage et al. [16], using inhibitors of proteinase and proteasome, showed that NF-κB is apparently involved in the expression of iNOS in LPS-stimulated rat alveolar macrophages. These findings indicate that NF-κB activation is important for the transcription of the iNOS gene in macrophages.

The heterodimeric NF-κB complex is sequestered in the cytoplasm as an inactive precursor complexed with an inhibitory protein, an IκB-like protein [17]. The most well characterized is the 37 kDa protein, IκBα [17]. The conversion of NF-κB into the active nuclear form, composed of 50 and 65 kDa subunits, is post-translationally induced by cytokines, LPS, TNF-α or oxidative stress. These stimulants seem to activate NF-κB by inducing the phosphorylation and release of IκBα [17,18], thereby allowing the rapid translocation of NF-κB from the cytoplasm to the nucleus, followed by DNA binding and gene expression [19].

Recently, in a large-scale screening test, α-benzoylamino-1,4-naphthoquinone (PPM-18), a chemically synthesized naphthoquinone compound (Figure 1), was found to possess a potent anti-inflammatory [20] and anti-sepsis activities. In this study we

Abbreviations used: EMSA, electrophoretic mobility-shift assay; iNOS, inducible NO synthase; LPS, lipopolysaccharide; NF-κB, nuclear transcription factor κB; PDTC, pyrrolidine dithiocarbamate; TNF-α, tumour necrosis factor α.

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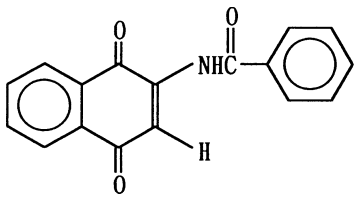

Figure 1 Chemical structure of PPM-18

investigated the effect of PPM-18 on the expression of iNOS by LPS in rat macrophages.

MATERIALS AND METHODS

Materials

PPM-18 (Figure 1) was chemically synthesized as described by Lien et al. [20]. All culture reagents were obtained from Gibco BRL (Gaithersburg, MD, U.S.A.). Bacterial LPS (*Escherichia coli*; serotype no. 0127:B8), calmodulin, cycloheximide, N^G-methyl-L-arginine, NADPH, sulphanilamide, naphthyl ethylenediamine, sodium nitrite, dithiothreitol, FAD, FMN, tetrahydrobiopterin and PDTC were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). L-[2,3,4,5-^{3}H]Arginine hydrochloride, horseradish peroxidase/luminol chemiluminescence reaction kit and isotope-labelled nucleotides, {[γ-^{32}P]ATP (3000 Ci/mmol), [α-^{32}P]ATP (5000 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol)} were obtained from Amersham (Little Chalfont, Bucks., U.K.). Recombinant constitutive NOS was obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.). The oligonucleotide corresponding to the palindromic NF-κB consensus sequence was synthesized in a cell synthesizer (Applied Biosystems, Foster, CA, U.S.A.). Rabbit polyclonal antibodies against p65, IκBα and iNOS were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Nucleic acid, protein molecular mass markers and Dowex AG 50W-X8 were purchased from Bio-Rad (Richmond, CA, U.S.A.).

Cell cultures

Rat alveolar macrophages were isolated from the lung by lavage 10 times with 5 ml of normal saline (0.9 %). Macrophages were then grown in Phenol Red-free Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal calf serum and an antibiotic mixture of penicillin (100 i.u./ml), streptomycin (100 μg/ml) and fungizone (1.25 μg/ml). Giemsa staining revealed that the alveolar cells were more than 98 % macrophages.

Measurement of nitrite

As a measure of NO production, nitrite, a stable metabolite of NO, in the medium was measured by the Griess reaction as described previously [21]. Briefly, 10^6 macrophages cultured in medium were stimulated with LPS in the presence or absence of PPM-18 for 24 h. The medium was then centrifuged and a 300 μl aliquot of the supernatant was mixed with 90 μl of Griess reagent [37.5 mM sulphanilic acid/12.5 mM *N*-(1-naphthyl)ethylenediamine dihydrochloride/6.5 M HCl] and incubated at room temperature for 10 min. The A_{540} of the mixture was read with an EL312e microplate reader (Bio-Tek). A series of sodium nitrite solutions with known concentrations were prepared as the standard, from which the amount of nitrite in the supernatant was calculated.

Measurement of nitrate plus nitrite

Nitrate and nitrite are the primary oxidation products of NO and therefore the nitrate plus nitrite (total nitrite) concentration was used as an indicator of changes in NO production *in vivo*. The blood sample was centrifuged (15000 ***g*** for 3 min) to remove cells and particles. Then the nitrate in the sample was enzymically converted to nitrite by a method described by Schmidt et al. [22] before measurement of the total nitrite content. Briefly, nitrate was stoichiometrically reduced to nitrite by incubation of sample aliquots (10 μl) for 15 min at 37 °C in the presence of nitrate reductase (EC 1.6.6.2) (1 unit/ml), NADPH (500 μM) and FAD (50 μM) in a final volume of 80 μl. When nitrate reduction was complete, the unused NADPH, which interferes with the subsequent nitrite determination, was oxidized with lactate dehydrogenase (100 units/ml) and sodium pyruvate (100 μM) in a final reaction volume of 100 μl and incubated for 5 min at 37 °C. Subsequently the total nitrite in the plasma was assayed by adding 30 μl of Griess reagent to the 100 μl sample.

Analysis of iNOS mRNA

Total cellular RNA was extracted once from 10^7 macrophages with an RNA extraction kit. Whole RNA was reverse-transcribed into cDNA and amplified for the iNOS gene with *Thermus aquaticus* (*Taq*) DNA polymerase in a thermal cycler (MiniCycler), as described previously [23]. A 540 bp product of reverse transcriptase–PCR was labelled with [α-^{32}P]dCTP, electrophoresed on 4 % (w/v) polyacrylamide gel and used to determine iNOS mRNA levels. The mRNA for the constitutive β-actin was examined as the reference cellular transcript. Autoradiography was performed at −80 °C, employing Kodak X-ray film with intensifying screens.

Western blot analysis

Macrophages grown and treated with LPS in the presence or absence of PPM-18 as described above were washed once with ice-cold PBS, and lysed in 1 ml of ice-cold lysis buffer [20 mM Tris/HCl (pH 7.4)/1 % (v/v) Triton X-100/100 mM EDTA/60 mM β-glycerophosphate/10 mM $MgCl_2$/0.1 mM NaF/2 mM dithiothreitol/1 mM Na_3VO_4/1 mM PMSF/5 μg/ml leupeptin/20 μg/ml aprotinin]. The lysates were then sonicated for 10 min and centrifuged at 14000 ***g*** at 4 °C for 1 min. An equal amount of protein (10–20 μg per lane) was subjected to SDS/PAGE [10 % (w/v) gel]. Proteins were transferred to a nitrocellulose membrane and incubated with TBST blocking solution [5 % (w/v) fat-free milk in PBS with 0.1 % (v/v) Tween-20] at 4 °C for 1 h. The membrane was then incubated with purified polyclonal rabbit IgG antibodies against murine antigens at 1:500 dilution (NF-κB p65, p50, IκBα and iNOS) overnight at 4 °C. After being washed, the membrane was treated with goat anti-(rabbit IgG) antibody conjugated with horseradish peroxidase, then illuminated with an enhanced chemiluminescence kit and exposed to Hyperfilm MP (Amersham).

Assay of NOS activity

To assess the effect of PPM-18 on constitutive NOS activity, recombinant nNOS and eNOS were used. NOS activity was measured in terms of the conversion of L-[^{3}H]arginine into L-[^{3}H]citrulline by the method described by Bredt and Snyder [24] with modifications. The production of L-[^{3}H]citrulline from L-[^{3}H]arginine was measured in the presence of NADPH (1 mM)/calmodulin (30 μM)/tetrahydrobiopterin (3 μM)/$CaCl_2$

(1 mM)//L-[^{3}H]arginine (1 μCi)/PPM-18 (10 μM) for 20 min at 37 °C in Hepes buffer (pH 7.5). To assess the effect of PPM-18 on iNOS activity, rat macrophage homogenates were incubated with the above reagent and EGTA (5 mM), but without Ca^{2+}. Reactions were stopped by dilution with 0.5 ml of ice-cold stop buffer containing 20 mM Hepes, pH 5.5, 2 mM EGTA and 2 mM EDTA. Reaction mixtures were applied to a Dowex AG 50 W-X8 column (Na^+ form, prepared from the H^+ form); L-[^{3}H]citrulline was eluted and measured by scintillation counting.

Electrophoretic mobility-shift assay (EMSA)

To assess the effect of PPM-18 on NF-κB–DNA binding, macrophages were incubated with PPM-18 for 60 min before stimulation with LPS (2 μg/ml) for 30 min. Nuclear extracts were then prepared as described [25]. The NF-κB oligonucleotide corresponding to the palindromic NF-κB consensus sequence (5′-GGCTCTCGGAAAGTCCCCTCTG-3′) was end-labelled with [α-^{32}P]dCTP, and purified on G-25 Sephadex columns. The nuclear extract (10 μg) was added to ^{32}P-labelled NF-κB oligonucleotide (10000 c.p.m.) in a buffer containing 4 μg of poly(dI-dC), 10 mM Hepes, pH 7.9, 40 mM KCl, 1 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM EDTA, pH 8.0, and 5% (v/v) glycerol (total volume 20 μl). To detect the DNA–protein complex, the mixture was subjected to non-denaturing PAGE [5% (w/v) gel] at 30 mA for 2 h in low ionic strength buffer (1 × TBE) at 4 °C. For supershift assays, anti-(NF-κB p65) antibody (0.3 μg) was added, before the addition of radiolabelled probe, to the nuclear extract derived from LPS-stimulated cells for 60 min. In some experiments, for specificity, PPM-18 or excess unlabelled NF-κB oligonucleotide (20 ng) was added directly to the nuclear extract before the addition of radiolabelled probe. After electrophoresis, the gel was dried and autoradiographed with an intensifying screen at −80 °C for 5 h.

Measurement of TNF-α release

TNF-α was assayed in the supernatant derived from macrophages by using the mouse TNF-α ELISA kit.

Mouse test

Balb/c mice (20–25 g) were purchased from the Animal Center of the National Taiwan University (Taipei, Taiwan). Mice were divided into four groups with 10 mice in each group and the drug was injected intraperitoneally. Mice were given 20 μl of DMSO (control) or PPM-18 (100 or 300 μg per mouse); 2 h later, three groups were given 2 mg of LPS per mouse. The mice were observed twice daily for death for 2 weeks. Death usually occurred within 5 days.

Haemodynamic measurements

Male Wistar rats (300–350 g; Biological Research Laboratories, Fullinsdorf, Switzerland) were anaesthetized with sodium pentobarbitone (40 mg/kg). The left carotid artery and the femoral vein were cannulated for blood pressure measurement and intravenous injection respectively. The arterial cannula was connected via a Statham pressure transducer to a Grass model 7 polygraph (Grass Instruments Co., Quincy, MA, U.S.A.) for measurement of arterial pressure. After a 30 min stabilization period, the basal value of blood pressure was recorded. To assess the effect of PPM-18 on LPS-induced hypotensive shock, after the end of the stabilization period, rats were treated with vehicle or PPM-18 (15 mg/kg, intravenously) for 20 min and then with LPS (10 mg/kg, intravenously) for 3 h.

Data analysis

Results are expressed as means ± S.E.M. A one-way analysis of variance was performed for multiple comparison, and, if there was significant variation between treatment groups, the mean values for an inhibitor were compared with those for the control by using Student's t test; P values of less than 0.05 were considered to be statistically significant.The n values indicate the numbers of independent experiments each conducted in duplicate.

RESULTS

Effect of PPM-18 on LPS-induced NO production and iNOS expression

Incubation of rat macrophages with LPS (2 μg/ml) for 24 h increased the production of nitrite (from 4 ± 2 to 56 ± 3 μM), a stable metabolite of NO, as measured by Griess reaction, indicating the production of NO. PPM-18 (0.1–10 μM) inhibited this nitrite increase in a dose-dependent manner, as well as the LPS-induced expression of iNOS protein (Figures 2a and 2b). This effect did not seem to be the result of a direct modification by PPM-18 (10 μM) of iNOS or other constitutive NOS (such as

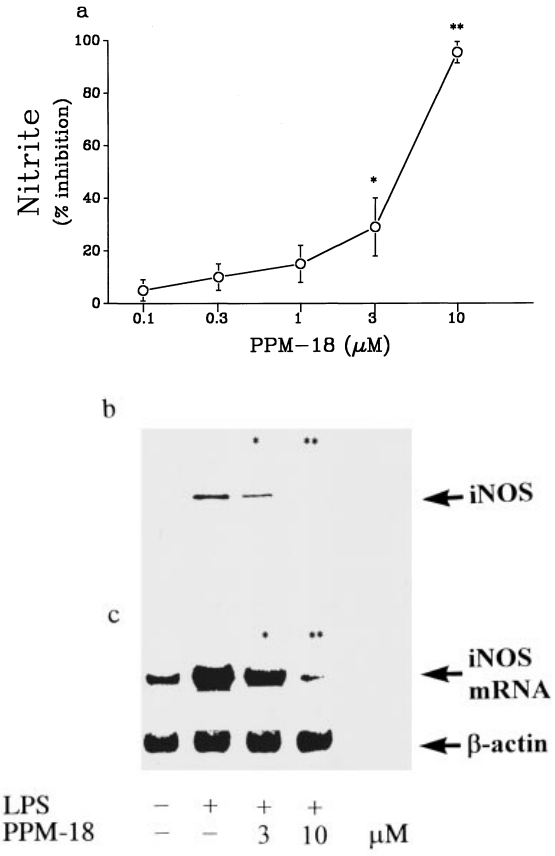

Figure 2 Inhibition of LPS-induced nitrite production, of and iNOS protein and iNOS mRNA expression

(**a**) Macrophages were stimulated with LPS (2 μg/ml) in the presence or absence of PPM-18 (0.1–10 μM) for 24 h. The stable metabolite of NO (nitrite) in the medium was measured by the Griess reaction. Cells were harvested at 24 h (**b**) for Western blot analysis and (**c**) at 6 h for iNOS mRNA analysis. β-actin was used as the internal control. Results in (**a**) are expressed as means ± S.E.M. ($n = 6$). Results in (**b**) and (**c**) are typical of four separate experiments. $^*P < 0.05$, $^{**}P < 0.001$ compared with LPS-treated cells.

Table 1 Effect of PPM-18 on activities of NOS forms

Recombinant eNOS from human umbilical vein (0.1 mg/ml), recombinant nNOS from rat brain (0.1 mg/ml) and iNOS from rat macrophage homogenates were incubated with DMSO (0.1%, solvent control) or PPM-18 (10 μM) for 20 min at 37 °C in Hepes buffer and their activities measured in terms of the amount of L-[^{3}H]arginine converted into L-[^{3}H]citrulline as described in the Materials and methods section. Results are expressed as means ± S.E.M. ($n = 4$).

	L-[^{3}H]Citrulline formed (c.p.m.)		
Treatment	eNOS	nNOS	iNOS
Control	2034 ± 346	810 ± 87	1327 ± 205
PPM-18	1875 ± 189	764 ± 10	1284 ± 345

Table 2 Effect of delayed addition of PPM-18 on nitrite production from macrophages

Macrophages were activated with LPS (2 μg/ml); PPM-18 (10 μM) was then added to the culture at the indicated time. After 24 h, nitrite concentration was measured. Results are expressed as means ± S.E.M. ($n = 4$). *$P < 0.001$ compared with LPS-treated cells.

Treatment	Nitrite (μM)
Resting	1.8 ± 0.9
LPS alone	57.3 ± 2.9
+ PPM-18 (2 h)	11.0 ± 3.6*
+ PPM-18 (4 h)	17.3 ± 3.0*
+ PPM-18 (6 h)	36.5 ± 2.0*
+ PPM-18 (12 h)	60.6 ± 2.2
+ PPM-18 (18 h)	57.0 ± 2.8

eNOS and nNOS) enzymic activity, as determined from L-[^{3}H]citrulline generation from L-[^{3}H]arginine (Table 1). PPM-18 (10 μM) also completely inhibited nitrate accumulation in LPS-stimulated macrophages (results not shown).

The finding that PPM-18 did not act directly on iNOS enzymic activity, yet still decreased the production of NO stimulated by LPS in rat alveolar macrophages, suggested that the target of PPM-18 might be the production of iNOS mRNA. This was examined. As shown in Figure 2(c), treatment of macrophages with LPS for 6 h raised the production of iNOS mRNA to a high level (lane 2). After preincubation of macrophages with PPM-18, the LPS-induced iNOS mRNA accumulation was inhibited (lanes 3 and 4).

Thus PPM-18 inhibited iNOS expression at the transcriptional level. Furthermore the inhibition of NO production by PPM-18 seemed to require the early presence of PPM-18 during the stimulation with LPS; in fact, when PPM-18 (10 μM) was added 12 h after the up-regulation of iNOS, no inhibition of nitrite production was observed (Table 2). These results also imply that once transcription started, PPM-18 was ineffective, i.e. PPM-18 seemed to act on the initiation of transcription.

Effect of PPM-18 on LPS-induced NF-κB activation

In the signal transduction pathway leading to iNOS expression in LPS-stimulated macrophages, NF-κB might be involved, as some studies have demonstrated [13,26]; the promoter for the iNOS gene seemed to contain the NF-κB binding consensus region [10]. Therefore the effect of PPM-18 on LPS-induced activation of NF-κB was examined by incubating macrophages with LPS for 30 min and analysing the NF-κB in the cytosol and

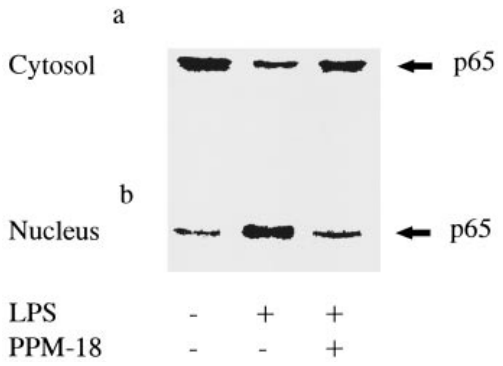

Figure 3 Effect of PPM-18 on the content of NF-κB p65 in the cytosol (a) or nucleus (b) of LPS-stimulated macrophages

Cells were incubated with PPM-18 (10 μM) for 60 min, and then with LPS (2 μg/ml) for another 30 min. The cells were lysed; nuclear extraction and Western blot analysis were as described in the Materials and methods section. Three separate examinations yielded similar results.

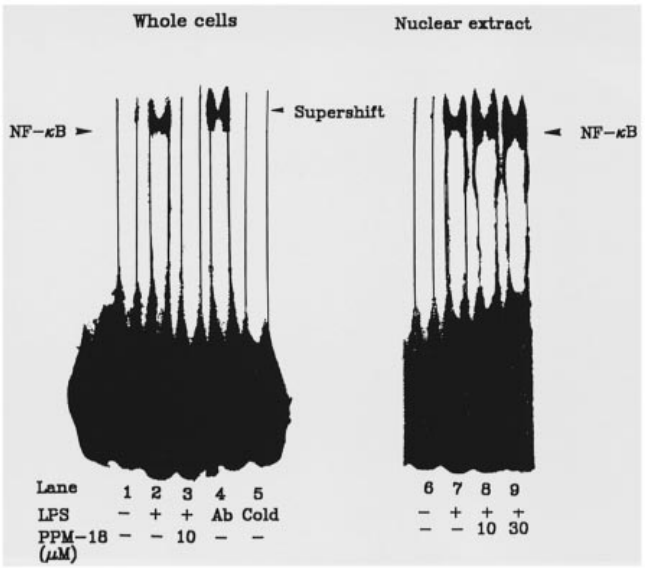

Figure 4 EMSA of the effect of PPM-18 on the DNA-binding activity of NF-κB

Macrophages were incubated with PPM-18 (10 or 30 μM) for 60 min before stimulation for 30 min by LPS (2 μg/ml), then lysed and tested (left panel) or added directly to the LPS-stimulated nuclear extract (right panel). The consensus nucleotide was then added to each test fraction, electrophoresed and autoradiographed as described in the Materials and methods section. The specificity of the binding was determined by adding anti-p65 antibody (0.3 μg; 'supershift') or excess unlabelled ('cold') NF-κB oligonucleotide (20 ng). This is a typical result of four separate experiments.

nuclear fraction by Western blot analysis. The amount of NF-κB in the nucleus seemed to increase more significantly than that in the cytosol after 30 min of stimulation with LPS. This LPS-induced increase of NF-κB (p65) seen in the nucleus was significantly inhibited by PPM-18 (10 μM) as shown in Figures 3(a) and 3(b). We next examined whether or not PPM-18 interfered with the binding of NF-κB to the DNA, by using EMSA with the consensus oligonucleotide for NF-κB binding. In untreated whole cells and the nuclear extract, a low-level NF-κB–DNA binding was detected (Figure 4, lanes 1 and 6). When

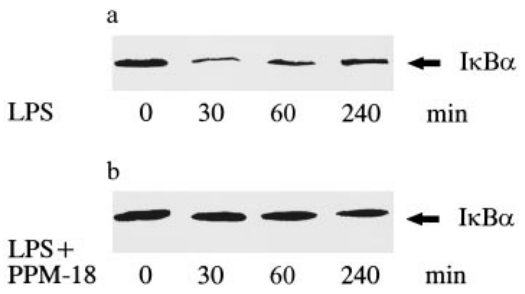

Figure 5 Effect of PPM-18 on the fate of IκBα

Macrophages were stimulated with LPS (2 μg/ml) in the absence (**a**) or presence (**b**) of PPM-18 (10 μM) for the indicated periods. IκBα in cytoplasm was detected by Western blotting as described in the Materials and methods section. Three separate experiments yielded similar results.

macrophages were incubated with LPS (2 μg/ml) for 30 min, the NF-κB–DNA binding activity was markedly elevated, both in the whole cell and in the nuclear extract (Figure 4, lanes 2 and 7). The shifted band shown was the specific complex formed by the binding of NF-κB to DNA, as the addition of anti-NF-κB antibody (p65) caused extreme gel retardation (supershift) (lane 4). Similarly, the addition of 100-fold excess unlabelled NF-κB oligonucleotide, which would compete for the binding of NF-κB also specifically abolished the NF-κB–nucleotide (radiolabelled) band (lane 5). Such NF-κB–DNA binding stimulated by LPS was markedly inhibited by co-incubation with PPM-18 (10 μM) in the whole cells (Figure 4, lane 3). However, when PPM-18 (10 or 30 μM) was added directly to the nuclear extract, inhibition of NF-κB–DNA binding was not observed (lanes 8–9). These results suggest that PPM-18 did not interfere the binding of NF-κB to DNA directly; rather, it acted on some events that might have been induced by LPS in the intact cells.

Stabilization of IκBα by PPM-18

Activation of NF-κB involves the release or degradation of the inhibitory subunit, IκBα, from a cytoplasmic complex that consists of IκBα, p65 and p50 [27]. We therefore examined the effect of PPM-18 on the fate of IκBα after treatment of macrophages with LPS. As shown in Figure 5(a), IκBα disappeared from the NF-κB complex in macrophages stimulated with LPS for 30 min. Treatment of cells with PPM-18 (10 μM) completely prevented the LPS-induced degradation of IκBα; the binding of NF-κB to DNA was also prevented (Figures 5b and 4). IκBα protein reappeared in the cytoplasm 1 h after stimulation with LPS. These results indicate that PPM-18 inhibited NF-κB activation by stabilization of IκBα.

Inhibition of TNF-α release by PPM-18

Because NF-κB seemed to be involved in macrophage activation and presumably in many other regulatory processes involving cytokine production, we tested whether or not PPM-18 could inhibit the expression of NF-κB-controlled release of cytokines such as TNF-α [28]. The TNF-α protein secreted into the cell medium was measured with ELISA; the result indicated that LPS induced a 6-fold increase in TNF-α levels and that this increase was inhibited by the addition of PPM-18 (10 μM) (Table 3).

Table 3 Effect of PPM-18 on LPS-induced release of TNF-α

Macrophages were incubated in the absence (control) or presence of LPS (2 μg/ml) with or without PPM-18 (10 μM) for 24 h. TNF-α was measured quantitatively with an ELISA kit. Values are expressed as means ± S.E.M. ($n = 4$). *$P < 0.001$ compared with LPS-treated cells.

Treatment	TNF-α concentration (pg/ml)
Control	434 ± 117
LPS	2711 ± 108
PPM-18 + LPS	260 ± 15*

Effect of PPM-18 on total plasma nitrite levels

Injection of rats with LPS (10 mg/kg) significantly increased the total plasma nitrite levels compared with the sham-operated rats at 360 min (9.9 ± 1.0 μM in sham-operated rats; 18.9 ± 1.3 μM in LPS-treated rats). This increase was significantly inhibited (down to 10.5 ± 1.1 μM) by pretreatment of rats with PPM-18 (15 mg/kg).

PPM-18 prevents LPS-induced lethal effect

LPS is an endotoxin and therefore the effect of PPM-18 on the toxic effect of LPS *in vivo* was examined. At the dose of 2 mg per mouse, LPS induced 90–95 % lethality within 5 days, but when PPM-18 (5 or 15 mg/kg) was given to the mouse 2 h before the administration of LPS, the lethality was decreased in a dose-dependent manner (Table 4). Under these conditions, PPM-18 conferred approx. 70 % protection when injected before the LPS treatment, and decreased protection when administrated later.

PPM-18 prevents delayed hypotension in endotoxic shock

At the end of the 30 min stabilization period, mean values for mean arterial pressure ranged from 120 ± 12 to 128 ± 10 mmHg. Administration of LPS (10 mg/kg, intravenously) induced a decrease in mean arterial pressure to 80 ± 10 mmHg within 5 min. At 3 h after LPS injection, there was a significant further decrease to 50 ± 7 mmHg. Rats pretreated with PPM-18 (15 mg/kg, intravenously) and subjected to LPS maintained a significantly higher mean arterial pressure at 3 h than LPS-treated controls (100 ± 8 compared with 50 ± 7 mmHg; $P < 0.001$).

Table 4 Effect of PPM-18 on LPS-induced lethal toxicity in mice

Mice were divided into four groups and injected intraperitoneally with DMSO (20 μl), LPS (2 mg) or PPM-18 plus LPS (2 mg). The mortality of mice was monitored twice daily for 2 weeks. No death was observed in those injected with DMSO alone or PPM-18 alone. Results are expressed as means ± S.E.M. (10 rats per group, $n = 4$). *$P < 0.001$ represents a significant difference compared with LPS-treated mice.

Treatment	Mortality (%)
Control	2 ± 2
LPS	95 ± 4
PPM-18 (5 mg/kg) + LPS	55 ± 5*
PPM-18 (15 mg/kg) + LPS	30 ± 7*

DISCUSSION

In the present study we demonstrate that PPM-18 suppresses the LPS-induced nitrite production in rat macrophages. This inhibition is not the result of a diversion of nitrite to nitrate or a direct modification of NOS enzymic activity; rather it results from blocking the expression of the iNOS gene at the transcriptional level. The concentration of PPM-18 necessary for inhibition of the LPS-induced NF-κB activation was parallel with that necessary for inhibition of nitrite production and iNOS protein or mRNA expression.

The expression of murine macrophage iNOS is largely regulated by transcription activation [9]. The presence of NF-κB-binding sequences in the iNOS promoter [10] and the requirement for nuclear translocation of NF-κB in iNOS induction [16] were supportive of the necessity for NF-κB activation in iNOS gene expression. Our results were consistent with this hypothesis; further, in the light of the hypothesis, they suggest the inhibition of iNOS induction by PPM-18 via the prevention of NF-κB activation. The activation and regulation of NF-κB are tightly controlled by IκB protein. Through non-covalent association, the IκB proteins mask the nuclear localization signal of NF-κB, thereby preventing NF-κB nuclear translocation. Our experiments on mRNA production clearly indicate that PPM-18 affects the initiation rather than mRNA synthesis itself. Also, once NF-κB is activated, PPM-18 is unable to inhibit its binding to DNA. Thus all observations indicate that the target of PPM-18 is IκBα: it blocks the activation of NF-κB by inhibiting the removal of IκBα, the inhibitor, from the NF-κB/IκBα complex. The results reported here support the hypothesis [15] that NF-κB is the principal transcription factor for iNOS transcription. A similar conclusion was reached recently by Griscavage et al. [16], who showed that inhibitors of proteinase and proteasome block the activation of NF-κB induced by LPS.

In the activation of NF-κB, reactive oxygen intermediates seem to have a role as messengers under many inducing conditions [29]. Antioxidants such as *N*-acetylcysteine or PDTC have also been shown to stabilize the NF-κB/IκBα complex via scavenging reactive oxygen species such as the superoxide anion, which might activate NF-κB [29,30]. This demonstrates that the proteolysis of IκBα is one of those controlled by reactive oxygen intermediates. However, PPM-18 has no antioxidant activity. Thus the stabilization of IκBα and thus inhibition of NF-κB activation by PPM-18 are probably not achieved through the scavenging of free radicals. How PPM-18 stabilizes IκBα is currently unknown. These are at least two possibilities: (1) PPM-18 might increase the phosphatase activity that dephosphorylates and thus stabilizes IκBα; or (2) PPM-18 might inhibit the activation of proteasomes.

Production of NO by iNOS is beneficial in fighting tumour cells, bacteria, fungi and parasites [2], but its overproduction can be harmful as shown in endotoxic shock [5,6] and inflammatory arthritis [31]. There is increasing evidence that overproduction of TNF-α during infection also leads to severe systemic toxicity and even death, e.g. TNF-α is a major factor in the development of septic shock after infection with Gram-negative bacteria [32]. The expression of the mouse TNF-α gene is regulated by NF-κB [32], and the product of the TNF-α gene also causes the activation of NF-κB [33] as well as inducing the expression of the iNOS gene [14]. Stamler et al. [30] demonstrated that expression of the TNF-α gene in response to LPS is strongly inhibited by the NF-κB inhibitor PDTC [14]. Therefore our results were consistent with this hypothesis, the suggested inhibition of TNF-α production by PPM-18 via the prevention of NF-κB activation.

The treatment of mice with PPM-18 did indeed reduce the lethal toxicity induced by LPS. In addition, pretreatment of rats with PPM-18 *in vivo* also inhibited the plasma concentration of nitrite and prevented the development of septic shock. The immediate and delayed hypotension observed in response to LPS in the model of endotoxaemia used in the present study are the result of enhanced NO formation, which is triggered by two distinct mechanisms: activation of the constitutive NOS and iNOS [33]. The finding that PPM-18 had no effect on the immediate hypotension in response to LPS (within 1 h), but significantly protected against the delayed [32] secondary decrease in mean arterial pressure (3 h after LPS treatment) supports the hypothesis that the beneficial haemodynamic effects of PPM-18 demonstrated in this study are due to the prevention of NOS induction. PPM-18 might also be of therapeutic benefit in other diseases associated with iNOS induction, such as chronic arthritis [31], chronic inflammation and transplant rejection [32].

In conclusion, we have demonstrated that PPM-18 inhibits the expression of iNOS via inhibition of the activation of NF-κB. PPM-18 might thus have potential for the treatment of endotoxaemia.

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